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DETERMINATION OF A NEW INOTROPIC AGENT IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new orally active inotropic agent, 6-[3,4-dihydro-3-methyl-(2H)-2-oxo-quinozolinyl)]-4,5-dihydro-3-(2H)-pyridazinone (I), is currently under investigation[1,2]. In support of clinical studies, a HPLC assay for the analysis of compound I in human plasma has been developed. The method involved a solid-phase extraction using C_{18} cartridge columns washed with methanol–water (20:80) and eluted with acetonitrile–water (70:30). The eluate was then extracted with dichloromethane. A reversed-phase alkylphenyl-bonded column was used as the analytical column. The mobile phase was a mixture of methanol, acetonitrile, 2-propanol and phosphate buffer (pH 4.6). A wavelength of 311 nm was used for detection. The limit of detection of the assay was 2 ng/ml, and the limit of quantitation was 5 ng/ml. A linear calibration range of 5 ng/ml to 1200 ng/ml was obtained with a correlation coefficient > 0.99. The precision and accuracy were evaluated by analyzing samples of three different concentrations ($n = 5$), 40, 200 and 800 ng/ml in plasma. The coefficients of variation and the differences from nominal values were less than 10%. The average recovery for 5, 50 and 100 ng/ml of analyte in plasma was about 90%. This assay has been applied to clinical studies with satisfactory performance and to plasma of different species in preclinical studies.

INTRODUCTION

A new, potent, orally active inotropic agent, 6-[3,4-dihydro-3-methyl-(2H)-2-oxoquinozolinyl)]-4,5-dihydro-3-(2H)-pyridazinone (I), is currently under preclinical and clinical investigation[1,2]. In support of these studies, a highly specific and sensitive method for the determination of the drug in biological matrices was required. This report describes a high-performance liquid chromatographic (HPLC) assay for analysis of compound I in human plasma. The assay involved both solid-phase and liquid–liquid extractions for sample purification prior to HPLC analysis. The method has been successfully applied to plasma from non-human species as well as in human pharmacokinetic studies.

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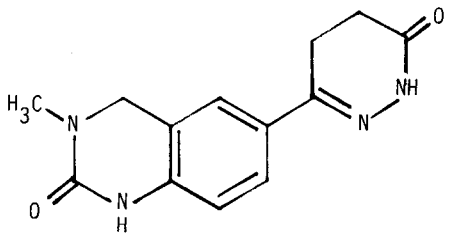

I

EXPERIMENTAL

Materials

HPLC-grade methanol, acetonitrile, 2-propanol and dichloromethane, and analytical-grade sodium phosphate (monobasic, ACS certified) were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). The 3-ml (500 mg) C_{18} Bond Elut cartridge columns were obtained from Analytichem (Harbor City, CA, U.S.A.). Pooled blank human plasma with sodium heparin as an anticoagulant was obtained from Biological Specialty Corp. (Lansdale, PA, U.S.A.). The analyte (I), and the internal standard [1,3-dihydro-3,3-dimethyl-5-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2H-indol-2-one], were synthesized at Rorer Central Research.

Preparation of the standard solutions

All stock solutions [10 μg/ml of compound I and 0.2 mg/ml of internal standard (IS)] were prepared with methanol–water (50:50). The working IS solution (1 μg/ml) was prepared by diluting the stock solution (0.2 mg/ml) with methanol–water (50:50). All other human plasma standards (0, 5, 10, 50, 100, 300, 600 and 1200 ng/ml of compound I) for standard curves were prepared by diluting the stock solutions with plasma.

Instrumentation

The HPLC system used consisted of a Waters (Milford, MA, U.S.A.) Model 510 dual-piston pump, a Waters intelligent sample processor (WISP), Model 710B and a Kratos (Ramsey, NJ, U.S.A.) Model 773 spectroflow UV detector. A Waters recording integrator, Model 730 data module, and a Nelson Analytical (Cupertino, CA, U.S.A.) Model 2600 data acquisition system, on a Compaq DeskPro 386 were also used in this study.

Extraction procedure

Frozen plasma samples, standards, or controls were placed in a warm water bath (40–45°C) for 10 min. To 1.00 ml of sample, standard or control in a 10 × 130 mm polypropylene tube, 100 μl of an internal standard solution (1 μg/ml) were added. After mixing, the samples were passed through a 3-ml (500-mg) C_{18} solid-phase extraction column with the aid of a vacuum. The extraction column was activated with one column volume of methanol and then water prior to performing the extraction. After the samples passed through the extraction column, the column was washed with

500 μl of methanol–water (20:80), and then eluted with two 500-μl volumes of acetonitrile–water (70:30).

To the 1 ml of eluate, 5 ml of dichloromethane was added. The solution was well mixed in a vortex mixer (approximately 0.5 min) and centrifuged at 1300 *g* (3000 rpm) for 5 min. The aqueous layer was aspirated and the organic layer was evaporated to dryness at 35°C under a gentle stream of nitrogen. The residue was dissolved in 100 μl of mobile phase, and a 40-μl aliquot was injected into the HPLC system.

Chromatography

An ES Industries (Marlton, NJ, U.S.A.) 150 × 4.6 mm I.D. column, packed with 5-μm particle size, 60-Å pore size, alkylphenyl reversed-phase material was used as the analytical column. An Upchurch Scientific (Oak Harbor, WA, U.S.A.) guard column, packed with Alltech–Applied Science (Deerfield, IL, U.S.A.) pellicular phenyl was used to protect the analytical column. The mobile phase consisted of methanol–2-propanol–acetonitrile–0.02 *M* sodium phosphate (monobasic) (15:6:7:72, v/v). A flow-rate of 1 ml/min and ambient temperature were used for the separation. The detection wavelength was 311 nm.

Quantitation and calibration

The standard curves were constructed by the peak-height ratios (I to IS) *vs.* concentration of the standards. Concentration values of samples were calculated by using non-weighted linear regression parameters from the standard curve. The calibration standards contained 0, 5, 10, 50, 100, 300, 600 and 1200 ng/ml of compound I in plasma. Due to the large concentration range, the calibration was divided into two curves; low range (0–100 ng/ml) and high range (0–1200 ng/ml). Concentrations of 100 ng/ml or below were interpolated from the low-range curve and any concentration value exceeding this was interpolated from the high-range curve.

Validation procedures

The method was validated to assess linearity, precision, sensitivity, accuracy, specificity, and viability of extracted samples on storage overnight. Three different concentrations (40, 200 and 800 ng/ml) of compound I-spiked human plasma samples (spiked control samples) were prepared in addition to the human plasma standards. On the first day of validation, human plasma standards were analyzed in triplicate along with the three concentrations of spiked controls samples ($n = 5$ for each concentration). Analyses of the standard and spiked control samples on subsequent days were used to assess within-day variability in the standard curve and the day-to-day precision and accuracy. Specificity of the analyses was demonstrated by comparing blank samples ($n = 3$) with I-spiked samples for each day of validation. Extraction recoveries for I and IS were determined by a peak-height comparison of extracted human plasma standards at 5, 50 and 100 ng/ml ($n = 3$ for each concentration) with the peak heights of comparable non-extracted aqueous standard (prepared with mobile phase).

Clinical samples

A single rising-dose safety study was performed in normal volunteers ($n = 16$, all male). For the determination of drug plasma levels, blood was drawn, following

a 10-mg and 15-mg oral dose (capsule). Subjects received active I at 4- to 5-day intervals to allow adequate washout of the previous dose. The plasma samples (heparinized) were collected at 0, 0.5, 1.5, 3.0, 6.0, 10 and 24 h after dosing, and were assayed, as described.

RESULTS AND DISCUSSION

A 2 ng/ml of analyte in plasma was found to be the detection limit which was determined by the signal-to-noise (S/N) ratio larger than five (Fig. 1). The standard curve was found to be linear between 5 and 1200 ng/ml, with correlation coefficients greater than 0.9957 for within-day analysis. A mean correlation coefficient $\pm$ S.D. of 0.9985 $\pm$ 0.0025 was obtained for the analyses ($n = 3$) of three days. The coefficient of variation (C.V.) of the slope (over three analysis days) was less than 3.3% (Table I).

Sample chromatograms (Fig. 1) of a human plasma blank and sample show the separation of the analyte and the internal standard. Although there is an unidentified peak at 6 min, the chromatogram showed no significant interferences, either with the analyte or with the internal standard. The retention times of compound I and IS are approximately 7.5 and 9.5 min, respectively.

The within-day precision of the plasma assay was assessed from five replicate extractions of control plasma standards at the three levels (Table II). The within-day C.V. ranged from 5.14 to 5.49% at concentrations of 40, 200 and 800 ng/ml in the plasma.

The day-to-day precision was determined over three consecutive days using the I-spiked plasma samples ($n = 5$ for each analysis day) of each concentration level (Table II). The C.V. of the interpolated I concentration were 1.56% for the low level (40 ng/ml), 2.97% for the mid level (200 ng/ml), and 2.35% for the high level (800 ng/ml).

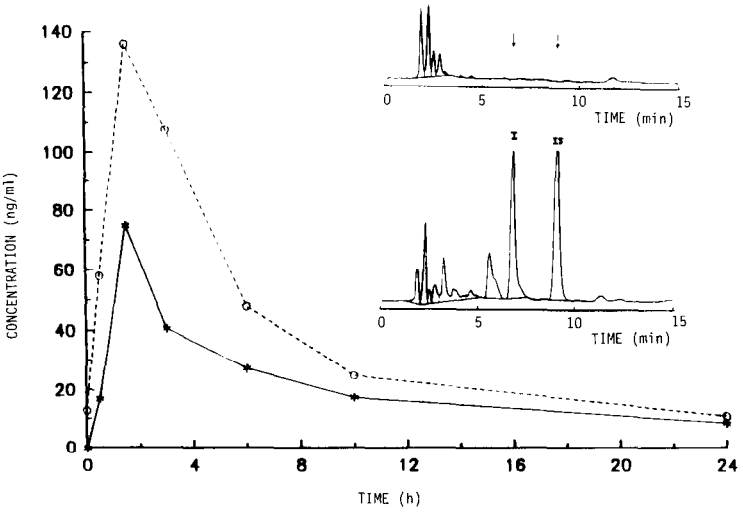

Fig. 1. Plasma concentrations of compound I as a function of time up to 24 h in a volunteer after a single oral dose (capsule) of 10 mg (lower curve) and 15 mg (upper curve). Chromatograms show the blank human plasma samples (upper chromatogram) and plasma samples drawn at 1.5 h after 10-mg capsule oral dose, plasma level is approximately 40 ng/ml. See text for the chromatographic conditions.

TABLE I

REGRESSION PARAMETERS FOR THREE DAYS ANALYSIS

Date	*Low-range curve (0–100 ng/ml)*			*High-range curve (100–1200 ng/ml)*		
	Slope	*Intercept*	*Correlation coefficient*	*Slope*	*Intercept*	*Correlation coefficient*
Day 1*	0.011453	0.011682	0.9993	0.011394	0.011987	1.0000
Day 2*	0.011682	0.0043388	0.9995	0.011278	−0.139970	0.9957
Day 3*	0.012209	−0.0046443	0.9996	0.011562	0.024990	0.9999
$\bar{x}$	0.011781	0.003792	0.9995	0.011411	−0.034331	0.9985
S.D.	0.000388	0.008177	0.0002	0.000143	0.091717	0.0025
C.V. (%)	3.29		0.02	1.25		0.25

* Single standard curve for each day.

Accuracy of the within-day analyses was determined by interpolation of the I-spiked plasma concentrations from the standard curve (Table II). The within-day difference between the mean and nominal values ranged between −2.75% and 2.00% for the control samples.

TABLE II

WITHIN-DAY PRECISION AND ACCURACY FOR CONTROLS INTERPOLATED FROM A STANDARD CURVE (A) AND DAY-TO-DAY PRECISION FROM THREE ANALYSIS DAYS (B)

MQL = Minimum quantitation level (n = 3).

	MQL, 5 ng/ml	*Low level, 40 ng/ml*	*Mid level, 200 ng/ml*	*High level, 800 ng/ml*
(A) Within-day				
1	4.85	37.7	198	795
2	4.70	36.0	190	738
3	4.36	40.8	206	850
4	–	39.8	206	834
5	–	40.2	219	791
$\bar{x}$	4.64	38.9	204	802
S.D.	0.25	2.0	11	44
C.V. (%)	5.39	5.14	5.39	5.49
Difference (%)	−7.20	−2.75	2.00	0.25
(B) Day-to-day				
Day 1		37.7	198	795
Day 2		38.9	209	832
Day 3		38.6	200	803
$\bar{x}$		38.4	202	810
S.D.		0.6	6	19
C.V. (%)		1.56	2.97	2.35
Difference (%)		−4.00	1.00	1.25

The recovery studies for compound I were performed in triplicate at three different concentrations (5, 50 and 100 ng/ml). The average recovery was approximately 92% ($n = 9$, S.D. = 13.6%, C.V. = 14.8%). The recovery for the IS at 1.0 μg/ml was 96.7% ($n = 9$, S.D. = 6.11%, C.V. = 6.32%).

This assay has been used for human plasma samples and different animal (rat, dog, and monkey) plasma samples with satisfactory performance. Fig. 1 presents the plasma profile of compound I in one patient who orally received active I (capsule) 10 mg or 15 mg once-a-day. Although, there is an unexplainable level of I at 0 h of 15-mg dose on this subject, it was not shown on most of the subjects. The incident is currently under investigation. Mean plasma concentrations of compound I in human subjects receiving a single 10-mg dose were between 5.74 and 48.0 ng/ml for the first 24 h after drug administration, suggesting that the sensitivity of the method is sufficient for monitoring the pharmacokinetic profiles after these doses.

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