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Journal of Chromatography B, 658 (1994) 202–206

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Determination of a novel and potent cyclic GMP phosphodiesterase inhibitor, 4-{[3,4-(methylenedioxy)-benzyl]amino}-6,7,8-trimethoxyquinazoline, in dog plasma by high-performance liquid chromatography

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First received 3 March 1994; revised manuscript received 29 March 1994

Abstract

A simple and accurate method for determining levels of 4-{[3,4-(methylenedioxy)benzyl]amino}-6,7,8-trimethoxyquinazoline, a novel cyclic GMP phosphodiesterase inhibitor, in dog plasma was developed. This method involves extraction with diethyl ether and reversed-phase high-performance liquid chromatography with ultraviolet detection. Regression analysis showed that the method was linear over the range 1.0–1000 ng/ml. The method was employed for the analysis of plasma samples in a preliminary pharmacokinetic study in beagle dogs.

1. Introduction

For the treatment of angina, nitrates represented by nitroglycerine and isosorbide dinitrate have often been used. Nitrates are well known to activate guanyl cyclase and increase the level of intracellular cGMP. This rise in cGMP level induces dilation of coronary arterics.

Cyclic nucleotides such as cAMP and cGMP are hydrolysed by phosphodiesterases (PDE). Five PDE isozymes are known [1] and one of them, cGMP-PDE (type V), hydrolyses cGMP specifically and is insensitive to Ca^{2+}-calmodulin. cGMP-PDE is a hydrolase of cGMP. Accordingly, if a selective inhibitor of cGMP-PDE can increase the intracellular cGMP level, the inhibitor has possibilities as a new type of drug for angina. However, no such inhibitor has been reported.

A newly synthesized compound, 4-{[3,4-(methylenedioxy)benzyl]amino}-6,7,8-trimethoxyquinazoline, ER-017996 (**I**) (Fig. 1), has a potent inhibitory action on cGMP-PDE isolated from porcine aorta ($IC_{50} = 0.36\ \mu M$), while its inhibitory activity towards other PDE isozymes was at least ten times weaker. Compound **I** also relaxed porcine coronary arteries precontracted with $PGF_{2\alpha}$ ($EC_{50} = 1.96 \pm 0.58\ \mu M$) [2].

So that **I** can be developed as a circulatory drug, its pharmacological action, pharmacokinetics and pharmacodynamics were investigated. This paper describes both a high-performance

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0378-4347/94/$07.00
SSDI 0378-4347(94)00186-9

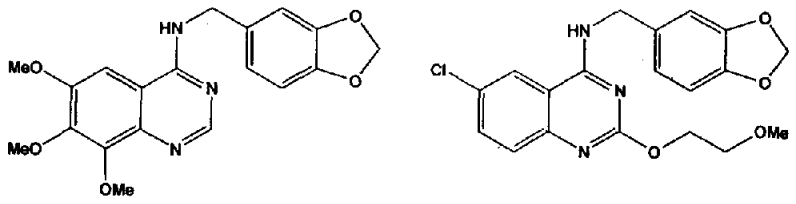

Fig. 1. Structures of I and II.

liquid chromatographic (HPLC) procedure for the determination of I levels in dog plasma and a preliminary pharmacokinetic study of the agent after intravenous and oral administrations to beagle dogs.

2. Experimental

2.1. Materials

Compounds **I** [2] and ER-021354 (**II**) (Fig. 1) [3] were synthesized in our laboratories. Both were confirmed to be more than 99% pure by HPLC. Other chemicals used were of analytical-reagent or HPLC grade.

2.2. Standard solutions

Compound **I** (100 mg) was dissolved in 100 ml of methanol and diluted with 0.1 *M* HCl to make a solution of 10 μg/ml. This solution was stored at 4°C for 1 month. Compound **II** (100 mg) was dissolved in 100 ml of methanol and diluted with 0.1 *M* HCl to make a solution of 1 μg/ml. This solution was also stored at 4°C for 1 month, and was used as the internal standard (I.S.).

2.3. Apparatus and chromatographic conditions

The HPLC system consisted of an LC-9A pump, equipped with an SCL-6B system controller, an SPD-6A UV spectrophotometric detector, a CTO-6A column oven, a C-R4AX Chromatopac and an SIL-6B autoinjector, all from Shimadzu (Kyoto, Japan). The mobile phase was acetonitrile–water–60% perchloric acid–sodium perchlorate monohydrate (380:620:1:5, v/v/v/w). The chromatographic column was a YMC Pack AM312 ODS (150 mm × 6 mm I.D., particle diameter 5 μm) obtained from Yamamura Chemical Labs. (Kyoto, Japan). The flow-rate, the wavelength for determination, and the temperature of the column were 1 ml/min, 330 nm and 40°C, respectively.

2.4. Calibration solutions

The standard solution of **I** was diluted with drug-free dog plasma to obtain plasma standard solutions at concentrations of 1.0, 2.5, 5.0, 10, 50, 100, 500 and 1000 ng/ml. The final volume of each plasma standard solution was 5 ml. For the preparation of daily calibration graphs, 3 ml of plasma standard solution at each concentration were prepared, the calibration graphs were prepared by analysing triplicate plasma samples at each concentration.

2.5. Assay procedures

To 0.8 ml of plasma sample was added 0.2 ml of I.S. solution, 0.2 ml of 1 *M* NaOH solution and 8 ml of diethyl ether and the mixture was shaken for 10 min. After centrifugation (3000 *g* for 5 min), the organic layer was removed and evaporated to dryness at 45°C under nitrogen. The residue was dissolved in 100 μl of 0.1 *M* HCl and 50 μl methanol and a 50-μl aliquot of the solution was injected into the chromatograph.

2.6. Recovery

The recovery of I was calculated by the following method. Plasma standards of I at concentrations of 5.0, 50 and 500 ng/ml were treated by the method described in Section 2.5, with 200 μl of 0.1 *M* HCl replacing the I.S. solution. After the organic layer had been evaporated, 200 μl of the I.S. solution and 50 μl of methanol were added to dissolve the residue. A 50-μl volume of the solution was injected into the chromatograph. The peak-area ratio of I to the I.S. was compared with that for the standard solution. The samples of the standard solutions for 5.0, 50 and 500 ng/ml plasma standards were prepared as follows. Standard solutions at I at concentrations of 50, 500 and 5000 ng/ml in 0.1 *M* HCl were prepared. Each standard solution (80 μl), 200 μl of I.S. solution and 50 μl of methanol were mixed and 50 μl of this mixed solution were injected into the chromatograph.

In the determination of the recovery for the I.S., 0.8 ml of drug-free dog plasma was treated by the method described in Section 2.5. The residue after the organic layer had been evaporated was dissolved by the addition of 80 μl of I standard solution (5000 ng/ml), 200 μl of 0.1 *M* HCl and 50 μl of methanol. This solution (50 μl) was injected into the chromatograph. The peak-area ratio of the I.S. to I was compared with that for the standard solution.

2.7. Calculations

The peak areas in the HPLC profiles were measured, and the ratio of the peak area of I to that of the I.S. was calculated. A calibration graph was constructed of peak-area ratio *versus* the concentration of I in the standards. The slope and intercept were calculated using weighted $(1/y)$ linear regression. The concentration of I in the experimental samples was calculated using the equation x (ng/ml) $= (y - b)/a$, where y is the ratio of I to the I.S. in an experimental sample and b (intercept) and a (slope) are constants generated by the linear regression analysis of the calibration data.

2.8. Animal study

Two beagle dogs (10 kg) were used; they were fasted for 18 h before and 6 h after drug administration and were allowed free access to water. The dosing interval was one week.

For intravenous (i.v.) administration, I was dissolved in ethanol; 3 ml of the ethanol solution containing 10 mg of I were added to isotonic sodium chloride solution, the final concentration of I being 1 mg/ml. The dose of I and the volume administered were 0.1 mg/kg and 0.1 ml/kg, respectively.

For oral administration (p.o), 3 mg of I were dissolved in 1.2 ml of ethanol, and 0.4 ml of this ethanol solution and about 10 ml of 0.1 *M* HCl were packed in a capsule. This capsule was administered orally, with 30 ml of water, to the beagle dogs. The dose was 0.1 mg/kg.

Blood samples (3 ml) were taken periodically from the right forefoot vein. The samples were centrifuged for 15 min at 3500 *g* to obtain plasma (1–1.2 ml), which was subjected to HPLC for the determination of I on the same day, according to the method described above.

2.9. Data analysis

The plasma concentration data for an individual animal after intravenous administration of I were fitted to the following equation:

$$C_p = A\,e^{-\alpha t} + B\,e^{-\beta t}$$

where C_p is the plasma drug concentration at time t, A and B are ordinate axis intercepts and α and β are the hybrid rate constants. The pharmacokinetic analysis of oral dosage forms was performed using model-independent methods, as the plasma concentration data did not fit the compartment model. The peak plasma concentration (C_{max}) and the time taken for attaining the peak concentration (T_{max}) were determined from the individual plasma concentration–time curves. The area under the plasma concentration–time curve (AUC), calculated by the trapezoidal rule, was added to the value of the plasma concentration at the time last detected

divided by the terminal elimination rate constant, which was calculated by the least-squares method with a semi-logarithmic scale.

Total plasma clearance (CL) was calculated by the following equation:

$$CL = dose_{i.v.}/AUC$$

The apparent volume of distribution at the steady state (V_{dss}) and the mean residence time (MRT) were obtained with the following equations:

$$V_{dss} = dose_{i.v.} \cdot AUMC/AUC^2$$

$$MRT = AUMC/AUC$$

where AUMC is the total area under the first moment of the drug concentration curve from zero to infinity.

The absolute bioavailability (BA) was calculated with the following equation:

$$BA = (AUC_{p.o.} \cdot dose_{i.v.})/(AUC_{i.v.} \cdot dose_{p.o}) \cdot 100$$

3. Results and discussion

Typical chromatograms for I obtained under the conditions described above are shown in Fig. 2. The retention times were approximately 10.6 min for I and 12.3 min for the I.S. The two compounds were well separated. The chromatogram of drug-free plasma showed no detectable interference from endogenous substances in the plasma.

Linear regression analysis gave slope, intercept and correlation coefficient values of $y = 0.005428x - 0.001166$, $r = 0.99998$, weight $= 1/y$ for the plasma calibration graph. The intra-day precision and accuracy were determined by analysing five replicate samples at each drug concentration. The precision and accuracy of this method, as shown in Table 1, ranged from 0.4% to 11.4%, and from 95.3% to 114.0%, respectively.

The inter-day and precision accuracy were determined by analysing triplicate samples at each standard concentration over five different days. The results from the calibration graph are given in Table 2. The precision at concentrations from 1.0 to 1000 ng/ml was 15.0% or better, which is good. The accuracy ranged from 93.5% to 120.0%. The interday precision and accuracy were acceptable.

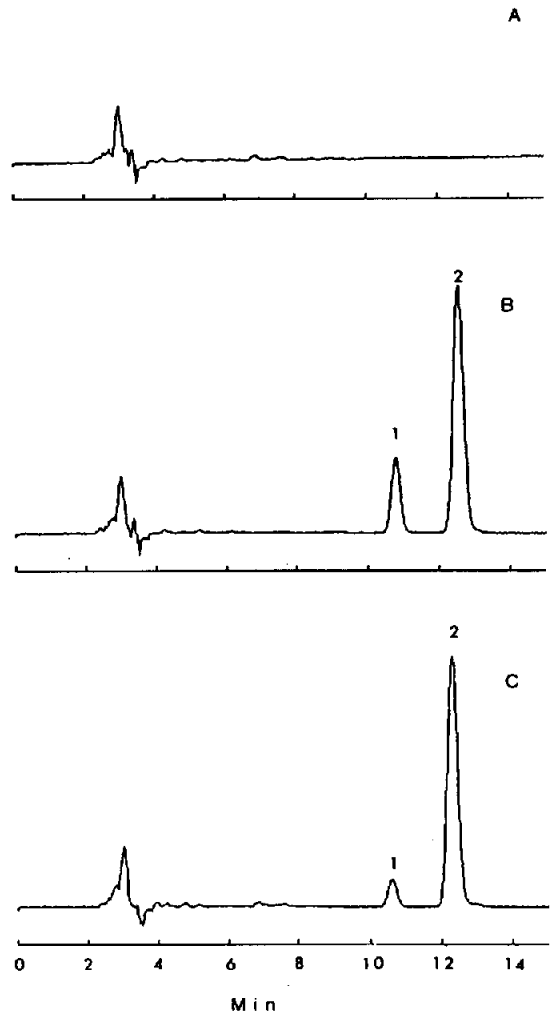

Fig. 2. HPLC of dog plasma containing (1) I and (2) the I.S. (A) Drug-free plasma; (B) plasma spiked with I at 50 ng/ml; (C) plasma from a dog 1 h after intravenous administration of 0.1 mg/kg of I (concentration of I, 18.5 ng/ml). Chromatographic conditions as described under Experimental. The detector was operated at 0.005 AUFS. A 0.8-ml sample of plasma was used for the assay.

Table 1
Intra-day precision and accuracy of the determination of I in dog plasma

Actual concentration (ng/ml)	Concentration found (ng/ml) (mean ± S.D., $n = 5$)	Precision (%)[a]	Accuracy (%)[b]
1.0	1.14 ± 0.13	11.4	114.0
2.5	2.49 ± 0.27	10.8	99.6
5.0	4.85 ± 0.21	4.3	97.0
10	9.53 ± 0.37	3.9	95.3
50	49.07 ± 0.71	1.5	98.1
100	98.86 ± 0.79	0.8	98.9
500	501.35 ± 1.77	0.4	100.3
1000	1001.44 ± 5.33	0.5	100.1

[a] (S.D./mean) · 100.
[b] (Concentration found/actual concentration) · 100.

The recovery of I at concentrations of 5.0, 50 and 500 ng/ml was 94.5 ± 0.8% (mean ± S.D., $n = 4$), 97.7 ± 2.1% ($n = 5$) and 89.4 ± 1.3% ($n = 5$), respectively. The recovery of the I.S. was 96.1 ± 1.5% ($n = 5$).

Fig. 3 shows the plasma concentration–time curves for I after intravenous and oral administration to beagle dogs at a dose of 0.1 mg/kg. All values are the means for two dogs. The plasma levels of I after intravenous administration appeared to decrease with time, with a biexponential pattern. The corresponding pharmacokinetic parameters are shown in Table 3.

Table 2
Inter-day precision and accuracy of the determination of I in dog plasma

Actual concentration (ng/ml)	Concentration found (ng/ml) (mean ± S.D., $n = 15$)	Precision (%)[a]	Accuracy (%)[b]
1.0	1.20 ± 0.18	15.0	120.0
2.5	2.50 ± 0.21	8.4	100.0
5.0	4.79 ± 0.39	8.1	95.8
10	9.35 ± 0.45	4.8	93.5
50	49.45 ± 1.24	2.5	98.9
100	99.90 ± 2.50	2.5	99.9
500	503.76 ± 9.06	1.8	100.8
1000	999.02 ± 18.28	1.8	99.9

[a] (S.D./mean) · 100.
[b] (Concentration found/actual concentration) · 100.

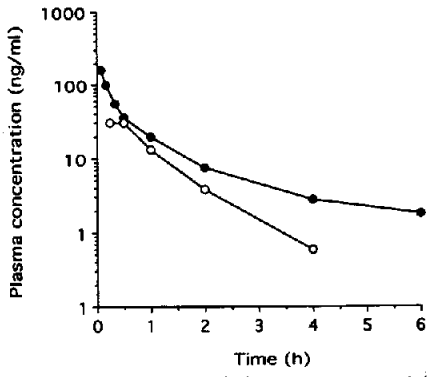

Fig. 3. Plasma levels of I after (●) intravenous and (○) oral administration of I at 0.1 mg/kg to beagle dogs. Each point represents the mean of values for two dogs.

Table 3
Pharmacokinetic parameters after intravenous and oral administration to beagle dogs at a dose of 0.1 mg/kg

Parameter	Intravenous	Oral
$t_{1/2}\beta$ (h)	1.38	–
MRT (h)	1.27	–
CL (ml/h/kg)	1163.3	–
V_{dss} (ml/kg)	1294.9	–
AUC (ng h/ml)	90.1	36.0
C_{max} (ng/ml)	–	31.8
T_{max} (h)	–	0.38
BA (%)	–	38.3

Each value represents the mean for two dogs.

C_{max}, T_{max}, AUC and BA on oral administration were 31.8 ng/ml, 0.38 h, 36.0 ng h/ml and 38.3%, respectively.

In conclusion, the proposed method, in which sample preparation is rapid, is simple, precise and accurate, and can be used in human and animal pharmacokinetic studies.

References

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