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Quantitative analysis of R-84760, a selective κ-opioid receptor agonist, in plasma by liquid chromatography with electrospray ionization tandem mass spectrometry

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Abstract

A sensitive method for monitoring R-84760, a selective κ-opioid receptor agonist, in plasma using liquid chromatography with electrospray ionization tandem mass spectrometry was explored. R-84760 and internal standard (I.S., d_8-R-84760) were extracted from human or various animal plasmas with ethyl acetate. The analysis was performed by the selected reaction monitoring method, and the precursor-product combinations of m/z 399–328 for R-84760 and m/z 407–328 for I.S. were chosen for quantification. The calibration curve was linear in the range 5–500 pg/ml, and the limit of quantification was 5 pg/ml using 1 ml of human plasma. Pharmacokinetic studies of R-84760 in rats, dogs, and monkeys were performed by this method. The plasma concentration of unchanged form after administration at a trace dosage amount was able to be monitored. Interspecies correlations of pharmacokinetic parameters obtained in animals were utilized to estimate pharmacokinetic behavior in humans. The results showed that it is possible to perform pharmacokinetic studies on R-84760 by this quantitative analysis.

Keywords: R-84760; LC-MS/MS; Quantification; Pharmacokinetic study; Animal scale-up

1. Introduction

R-84760 is a novel κ-opioid receptor agonist, whose chemical structure is (3*R*)-3-(1-pyrrolidinylmethyl)-4-[(1,*S*)-5,6-dichloro-1-indancarbonyl]-tetrahydro-1,4-thiazine hydrochloride [1,2] (Fig. 1). R-84760 has a higher affinity to κ-opioid receptor [3] than to μ or δ-opioid receptor, and it has potent antinociceptive activity. Its antinociceptive potency is 360 and 370 times higher than U-50488 [4] and morphine, respectively, in the phenylquinone writhing test [1]. Therefore, it has been expected that its clinical dosage to gain an antinociceptive effect will be very low.

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However, its low dosage will make it difficult to monitor the plasma concentration for pharmacokinetics. Because UV absorption of R-84760 is very weak, it was impossible to measure trace amount of unchanged form in plasma by conventional HPLC method. Furthermore, R-84760 has no functional groups to derive for purpose of high sensitivity so it is difficult to be determined by GC-MS or immunological assay. Receptor binding assay was also tried, but it was impossible to establish a high sensitive analysis.

Liquid chromatography with atmospheric pressure ionization tandem mass spectrometry has been used practically for the quantitative analysis of drugs [5–7], other organic compounds [8,9], or peptides [10] for the improvement of ion source, collision induced dissociation, and system suitability. Therefore, we tried to measure of R-84760 by liquid chromatography with electrospray ionization (ESI) tandem mass spectrometry, and applied for the pre-clinical study of R-84760.

2. Experimental

2.1. Materials

R-84760 and d_8-R-84760 ((3R)-3-(1-d_8-pyrrolidinylmethyl)-4-[(1,S)-5,6-dichloro-1-indancalbonyl]-tetrahydro-1,4-thiazine hydrochloride; internal standard, I.S.) (Fig. 2) were synthesized in the Medicinal Chemistry Research Laboratories, Sankyo (Tokyo, Japan). Other reagents and solvents were of analytical grade and were used without further purification.

2.2. Preparation of standard and I.S. solutions

R-84760 and I.S. were dissolved in methanol, 100 μg/ml each. These stock solutions were stable at 4°C for 1 year. These solutions were used as the R-84760 and I.S. standard solutions for the preparation of a calibration curve.

2.3. Pretreatment

One hundred μl of I.S. solution (0.5 ng/ml), which was stock solution diluted with saline, and 1 ml of saline were added to 1.0 or 0.1 ml of serum, and the mixture was extracted with 5 ml of ethyl acetate [11]. After shaking for 10 min and centrifuging, the organic layer was transferred into another tube and evaporated in vacuo at 50°C. The residue was re-dissolved with 100 μl of HPLC mobile phase, and that solution was filtered with a chromato disk type filter (GL Science, Tokyo, Japan). 10 μl of the sample was injected into the LC-ESI/MS/MS system.

2.4. LC-MS/MS condition

The LC system was used of a Model HP1050 LC system (Hewlett-Packard, Silicon Valley, USA), consisting of a low-pressure pump, an autosampler, and a semi micro column (Capcell Pak UG-120, C18, 150 × 2 mm I.D., Shiseido, Yokohama, Japan). The mobile phase was composed of an ICPP-MS3 (GL Science, Tokyo, Japan), a volatile counter ion reagent: water: acetonitrile (0.05:58:42, v/v/v), and the flow rate was 0.2 ml/min.

A triple-stage quadruple mass spectrometer (TSQ-700, Thermoquest, San Jose, CA, USA) equipped with an API source (Thermoquest) was used for LC-MS/MS. The API source was fitted with an ESI inlet, and the positive-ion selected reaction monitoring (SRM) mode was chosen for the quantification.

The ESI voltage was set at −4.5 kV, and capillary temperature was 250°C. The sheath and auxiliary gas pressure were 60–35 psi, respectively. The collision gas pressure and the collision offset voltage were 2 mTorr and −25 eV, respectively. The scan ranges switched over at 0.8 s intervals.

The peak integration was calculated by peak-area ratios obtained from SRM of R-84760 (m/z 399–328)/I.S. (m/z 407–328).

2.5. Calibration and validation

Samples for the calibration curve were prepared by adding 100 μl of the diluted standard solutions of R-84760 in saline to the control serum, and they were treated the same as described above.

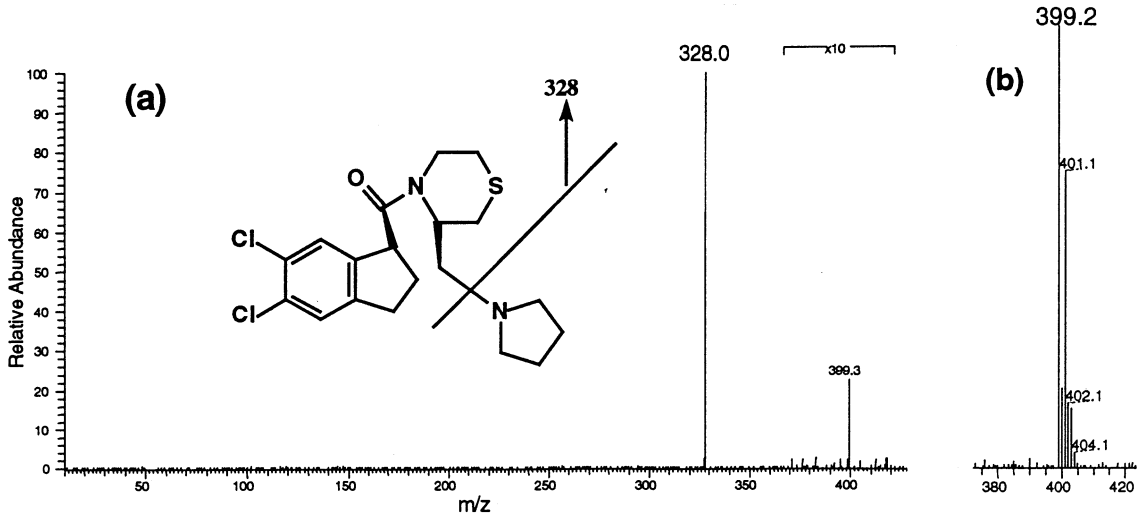

Fig. 1. ESI-MS, ESI-MS/MS spectra of R-84760 (50 ng/inj): (a) ESI-MS/MS spectrum of R-84760 (m/z 399), its fragment pattern; (b) part of ESI-MS spectrum of R-84760.

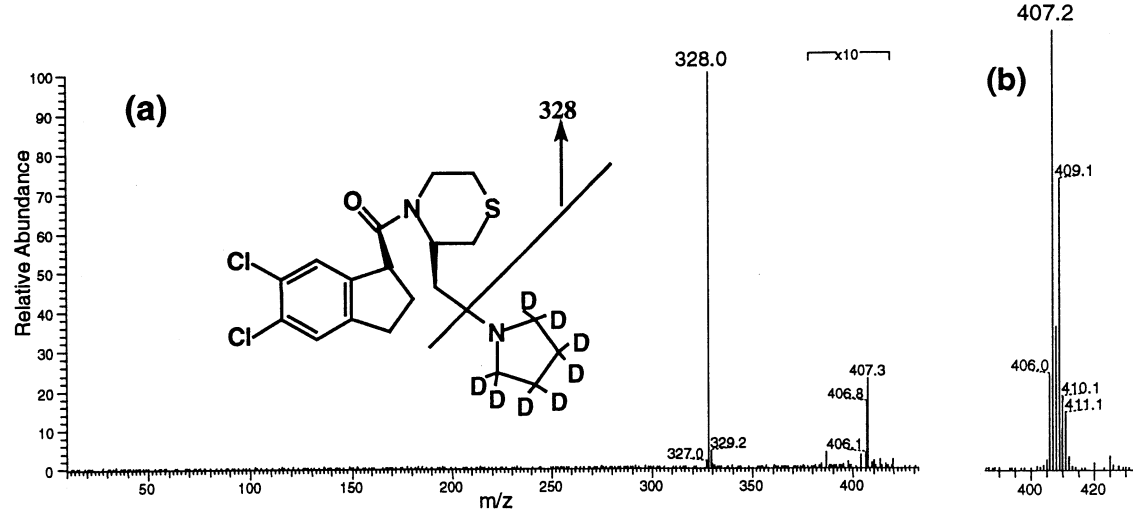

Fig. 2. ESI-MS, ESI-MS/MS spectra of d_8-R-84760 (50 ng/inj): (a) ESI-MS/MS spectrum of d_8-R-84760 (m/z 407) and its fragment pattern; (b) part of ESI-MS spectrum of d_8-R-84760.

The standard solutions were added at the concentrations in the range from 50 to 5000 pg/ml, corresponding to the plasma concentrations in the range from 5 to 500 pg/ml when using 1 ml of plasma.

2.6. Pharmacokinetic studies in rats, dogs, and monkeys

Animals were housed for at least 1 week in an animal room at controlled temperature (23 ±

1°C), with artificial lighting (07:00–18:00). Food and water were available ad libitum.

Male rats (F344) were obtained from Imamichi Institute for Animal Reproduction (Ibaragi, Japan). Rats 7 weeks of age and weighing approximately 220 g were fasted overnight before dosing.

Male dogs (beagles), which were bred in the Laboratory Animal Science and Toxicology Laboratories, Sankyo (Shizuoka, Japan), were used. Dogs 1 to 2 years of age and weighing approximately 10 kg were used. Male monkeys (*cynomolgus*) were obtained from Japan SLC (Shizuoka, Japan). Monkeys 5–8 years of age and weighing ≈ 3 kg were used.

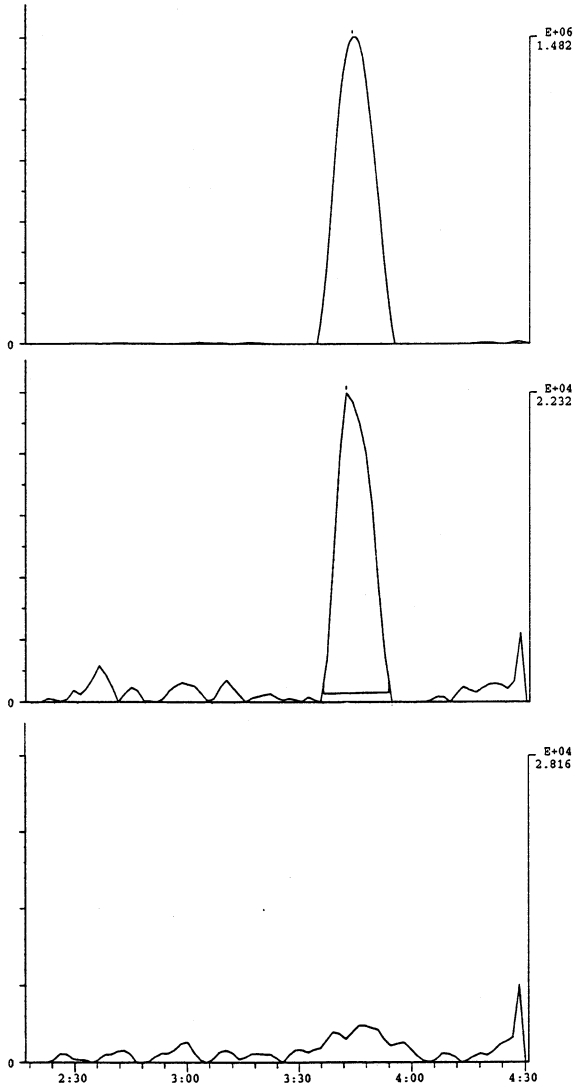

Fig. 3. Typical chromatograms of R-84760 in human plasma obtained by SRM at m/z 407 → 328 for I.S. (top) and at m/z 399 → 328 for R-84760 (center and bottom). (top) 500 pg/ml of I.S., (center) 5 pg/ml of R-84760, (bottom) blank serum.

After single subcutaneous administration of R-84760 (1, 5, 50, 100, 2000 μg/kg in saline) to rats ($n = 5$), blood samples were collected at 0.25, 0.5, 1.5, 3, and 6 h, with heparinized syringes under anesthesia.

Immediately after each of the multiple subcutaneous administrations of R-84760 (2, 20, 2000 μg/kg in saline) to dogs ($n = 6$) at once a day for 4 weeks, the animals were fed. Blood samples were collected at 0.5, 1, 2, 4, 6, and 24 h on the first and final days, with heparinized syringes without anesthesia.

Immediately after each of the multiple subcutaneous administrations of R-84760 (200, 2000 μg/kg in saline) to monkeys ($n = 2$) at once a day for 2 weeks, the animals were fed. Blood samples were collected at 0.25, 0.5, 1, 2, 6, and 24 h on first and final days, with heparinized syringes without anesthesia.

The collected blood was centrifuged at 12 000 g for 3 min, and the obtained plasma was stored at −20°C until analysis.

3. Results

3.1. Spectra of R-84760 and I.S.

MS spectra of R-84760 and I.S. are shown in Figs. 1 and 2. Each base peak of R-84760 and I.S. was m/z 399–407, corresponding to the protonated molecule $[M + H]^+$. The product ion mass spectra of these protonated molecules of R-84760 and I.S. indicated the presence of the most intense product ion at m/z 328, due to the loss of the pyrrolidine ring side. Therefore, the precursor-product combinations of m/z 399–328 for R-84760 and m/z 407–328 for I.S. were chosen for quantification.

3.2. Calibration and validation data

Typical chromatograms of extracts of R-84760 and I.S. spiked human plasma (1 ml) are shown in

Table 1
Intra-day precision and accuracy data for the analyses of R-84760 in human plasma ($n = 4$)

Nominal concentration (pg/ml)	Mean (pg/ml)	SD	CV (%)	Accuracy (%)*	
15	14	2	14.29	93.33	
100	103		3	2.91	103.00
500	493		15	3.04	98.60

* Calculated as (mean calculated concentration/nominal concentration) × 100.

Table 2
Inter-day precision data for the analyses of R-84760 in human plasma[a]

Nominal concentration (pg/ml)	Intra-day		Inter-day	
	Initial mean (pg/ml)	CV (%)	Mean (pg/ml)	CV (%)
15	18	8.33	16	16.56
100	94	4.63	98	5.57
500	501	3.11	493	2.74

[a] $n = 4$. $n = 8$, performed over a period of five days.

Fig. 3. Retention time of both analytes was within 4 min, and the analysis finished at 4.5 min. No other peak was observed from both chromatograms, and I.S. did not influence the mass chromatogram of R-84760, because of its good isolation and purity. The limit of quantification (LOQ) of R-84760 was 5 pg/ml plasma.

The calibration curves demonstrated good linearity from 5 to 500 pg/ml, and correlation coefficient was $r^2 = 0.998$.

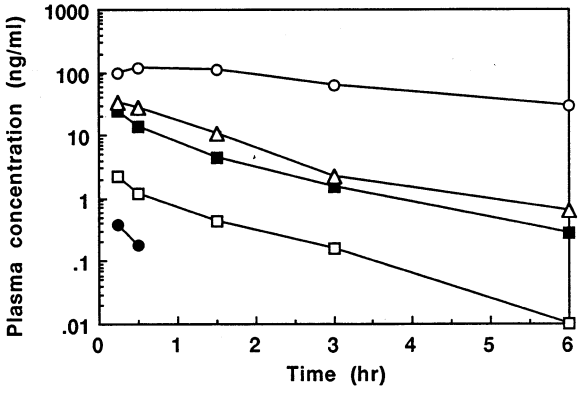

Fig. 4. Time-plasma concentration profile of R-84760 after single subcutaneous administration (○: 2 mg/kg, △: 100 μg/kg; ■: 50 μg/kg; □: 5 μg/kg; and ●: 1 μg/kg) to rats ($n = 5$).

The result of assay precision is shown in Tables 1 and 2. Low, middle, and high concentration standard samples were chosen for the intra and inter-assay precision. The inter-assay interval was 5 days. The coefficient of variation at each concentration was within 15% for the intra-assay precision, and was also for the inter-assay precision, except the low concentration, which was close to the LOQ.

3.3. Pharmacokinetic study

The method was applied to the measurement of R-84760 after administration to rats, dogs, and monkeys for the pharmacokinetic study. The analytical method for those animals was performed using the same conditions used for humans, except for the plasma volume (0.1 ml).

The plasma concentration-time profiles after administration of R-84760 to rats are shown in Fig. 4. When the plasma samples were collected at 0.25 and 0.5 h at the minimum dosing (1 μg/kg), R-84760 in the plasma sample could be readily detected.

Pharmacokinetic parameters in rats, dogs, and monkeys are shown in Table 3. Relationship between dose and *C*max, or dose and AUC are summarized in Fig. 5. Both AUC and *C*max

Table 3
Pharmacokinetic parameters of R-84760 in plasma

Animal	Day	Dose (μg/kg)	*n*	AUC (0–6 h) (ng h/ml)	AUC (0–24 h) (ng h/ml)	*C*max (ng/ml)
Dog	1st	2	6	14.1	20.5	6.9
		20	6	78.4	116.8	38.9
		2000	6	3251.0	4864.6	1831.0
	28th	2	6	10.7	21.5	6.4
		20	6	130.0	257.0	47.2
		2000	6	2074.3	4411.4	1131.0
Monkey	1st	200	2	281.6	413.2	222.9
		2000	2	3575.2	5109.8	1630.3
	14th	200	2	377.5	535.0	416.3
		200	2	3690.2	4507.2	2107.4
Rat	1st	1	5		0.4	
		5	5	2.2		2.2
		50	5	23.9		24.5
		100	5	45.5		32.7
		2000	5	433.0		118.0

values for the unchanged form were found to be linear against dose in the three animal species, and the order of these value was monkey = dog > rat. There were no differences between the concentrations on the first and final days at multiple administrations to dogs or monkeys.

4. Discussion

4.1. On the synthesis of I.S.

R-84760 has many stable isotopes because of its two chloric group. If 4 deuterium was introduced to R-84760 for I.S., (^{37}Cl,^{37}Cl)-R-84760 would interfere with the measurement of I.S. Therefore, it is necessary to separate the molecular weight of I.S. from that of R-84760 over 5 mass units in general. Thus far, it is a general idea on using LC-MS method. In the case of the SRM method, it is significant to consider the selected product ion. It is important to select a moiety that does not include chlorine in the labelled position. In the case of R-84760, product ion obtained from the loss of the pyrrolidine ring side includes two chlorine. Therefore, a stable isotope should be introduced to the pyrrolidine ring. For example, 3 or 4 deuterium labelled R-84760 is able to be separated from (^{37}Cl,^{37}Cl)-R-84760 in order to isolate by Q3MS.

As it turned out, 8 deuterium labelled R-84760 was able to be synthesized. However, it showed that it was significant to analyze the fragment pattern before synthesizing stable isotope labelled I.S.

4.2. Pharmacokinetic extrapolation to human

It was possible to estimate pharmacokinetic behavior in humans to utilize interspecies correlations of pharmacokinetic parameters obtained in animals [12,13]. It was useful to design the Phase-I clinical study of R-84760. Excellent allometric relationships were obtained between model-independent pharmacokinetic parameters, i.e. total body clearance (CLtotal) or apparent steady state volume of distribution (Vdss), and body weight (BW) in three kinds of animals, as follows.

$$\text{CLtotal (ml/min)} = 16.08 \times (\text{BW})^{0.5425},\ r = 0.985$$

$$V\text{dss (L)} = 2.209 \times (\text{BW})^{0.8764},\ r = 0.987$$

Appropriate conversion of actual sampling times and plasma concentrations in animals based on the complex-Dedrick theory [14] yielded predictions of concentration (Cp) versus time (T) profiles after subcutaneous doses in humans, which were satisfactorily described by the bi-exponential equation, as follows:

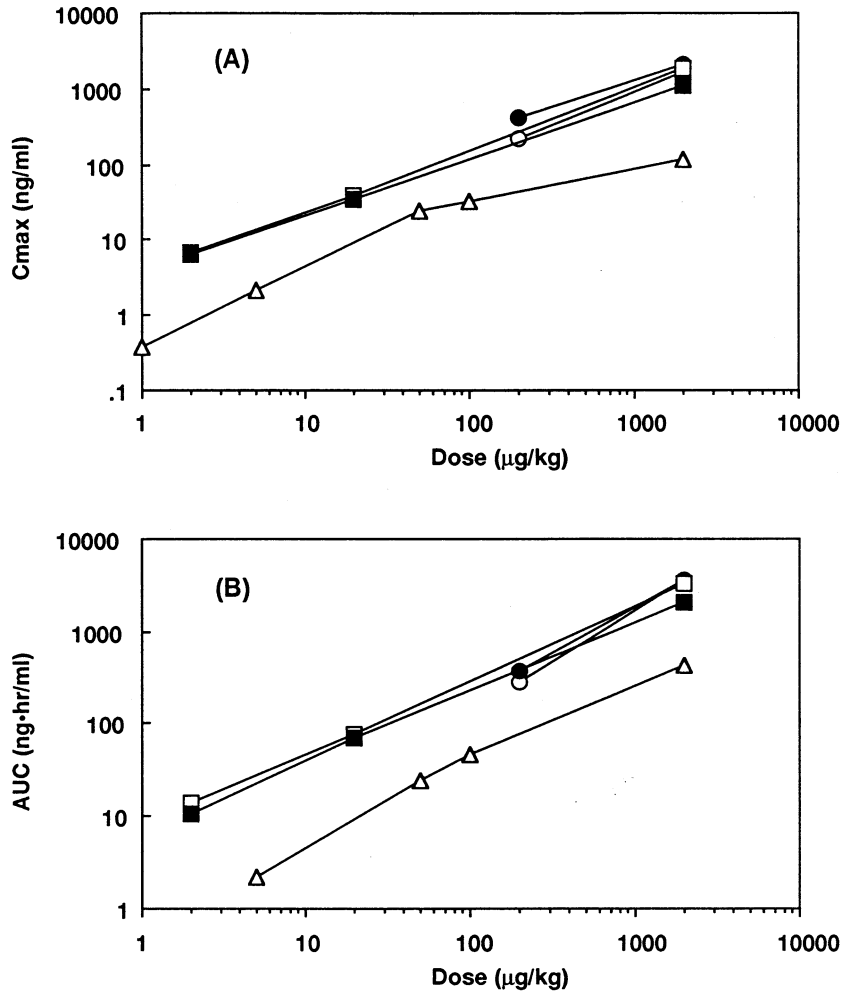

Fig. 5. Relationship between dose and *C*max (A) and between dose and AUC (B) ○: 1st dose to monkeys ($n = 2$); ●: 14th dose to monkeys ($n = 2$); □: 1st dose to dogs ($n = 6$); ■: 28th dose to dogs ($n = 6$); and △: 1st dose to rats ($n = 5$).

$$Cp \text{ (ng/ml)} = 48.0 * \exp(-0.6545 * T) + 6.96 * \exp(-0.0592 * T)$$

T: time after a 20 μg/kg dose of R-84760 (h)

The peak plasma concentration (*C*max) would exceed 5 pg/ml, the LOQ with this SRM method, after a subcutaneous dose of 1 μg/body in humans, which allows us to perform pharmacokinetic studies on R-84760.

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