Arch. Pharm. Res. Vol.19, No.6, pp. 554-558, 1996

High Performance Liquid Chromatographic Assay of a New Fluoroquinolone, LB20304, in the Plasma of Rats and Dogs

Mi-Kyeong Seo, Yi-Na Jeong, Hoon-Joo Kim, In-Chull Kim and Yong-Hee Lee*

LG Chem Biotech Research Institute, Moonji-Dong 104-1, Yu Song, Taejon 305-380, Korea

(Received June 24, 1996)

High-performance liquid chromatographic method was developed for the determination of LB 20304 (compound 1) in the plasma of rats and dogs. The analyte was deproteinized with 1 volume of methanol and 1/2 volume of 10% zinc sulfate, and the supernatant was injected onto a reversed-phase HPLC column. The mobile phase was a mixture of 24 parts of acetonitrile and 76 parts of 0.1% trifluoroacetic acid. The flow rate was 1 ml/min, and the effluent was monitored by fluorescence detector at an excitation wavelength of 337 nm and an emission wavelength of 460 nm. The retention time of compound 1 was 6.3 min. The assay of compound 1 was linear over the concentration range of 0.2-100 μg/ml in the plasma of rats and dogs. The lower limit of quantification was 0.2 μg/ml using 100 μl of plasma with a 97-99% accuracy and a 12-14% precision. In the 0.5, 5, and 50 μg/ml quality control samples, the intra- and inter-day accuracy were 88-95% and 88-97%, whereas intra- and inter-day precision were 0.5-6.6% and 0.2-9.3%, respectively, in the plasma of rats and dogs. The recoveries were 68-71% independent of concentration and species in the plasma. No interferences from endogenous substances were observed. Taken together, the above HPLC assay method by deproteinization and fluorescence detection was suitable for the determination of compound 1 in the preclinical pharmacokinetics.

Key words : HPLC assay, LB20304, Fluoroquinolone, Plasma

INTRODUCTION

LB20304a (mesylate salt of compound 1, 7-(3-aminomethyl-4-methyloxyiminopyrrolidin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-1,8-naphthylidine-3-carboxylic acid methane sulfonic acid) is a new quinolone antibacterial agent (Fig. 1). It showed a broad spectrum activity, particularly potent antibacterial activities against gram-positive bacteria *in vitro* efficacy study (Kim *et al.*, 1996). The improved activity against gram-positive pathogens, particularly *Streptococci* and *Staphylococci*, suggests a significant therapeutic potential for this compound.

Pharmacokinetic studies of this compound in animals require sensitive and reproductive analytical methods for the quantification of the drug in biological fluids, such as plasma, urine, bile, and tissue homogenates. For the quantification of compound 1 in the plasma of rats and dogs, a simple isocratic high-performance liquid chromatographic (HPLC)-fluorescence detection assay method using an ion-pair chromatography on a reversed-phase column has been developed. This method was successfully applied for the preclinical pharmacokinetics of compound 1.

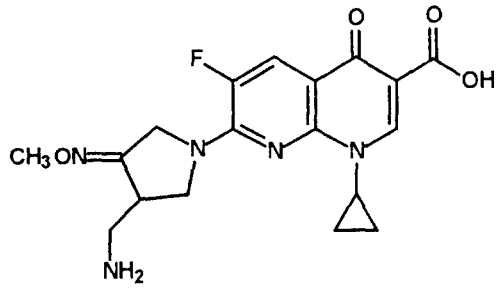

Fig. 1. The structure of LB20304a

MATERIALS AND METHODS

Chemicals and reagents

LB20304a (Lot No.: Q002, purity: 98%) was synthesized at LG Chem Biotech Institute (Tae-jon, Korea). Trifluoroacetic acid (TFA) and zinc sulfate were obtained from Sigma (St. Louis, MO, USA). Acetonitrile and methanol (Burdick and Jackson,

Correspondence to: Yong-Hee Lee, Ph.D. LG Chem Biotech Research Institute, Moonji-Dong 104-1, Yu Song, Taejon 305-380, South Korea

Muskegon, MI, USA) were of HPLC grade, and all other reagents were of analytical grade. Deionized water was purified using a Milli-Q filter system (Millipore, Milford, MA, USA).

Preparation of standard solutions

Stock solution (1 mg/ml) of compound 1 was prepared by dissolving LB20304a in methanol in a 14-ml polystyrene tube (Becton Dickinson Labware, Franklin Lakes, NJ, USA). For the preparation of calibrator, diluted working solutions of compound 1 (0.2, 0.5, 1, 5, 10, 50, and 100 μg/ml) were made with methanol in a polystyrene tube. A 100 μl of the corresponding working solutions was treated over a four-day period (n=4) to construct 4 calibrators. Response factors were calculated by dividing peak area of the compound 1 by their added concentrations (μg/ml), and used for the back-calculation of evaluated concentration in calibration standards and quality control samples. Lower limit of quantification (LOQ) was determined as the lowest concentration on the calibration standard with acceptable accuracy (above 80%) and precision (within 20%) (Shah et al., 1992). The accuracy (mean) and the precision (C.V., coefficient of variation) of the LOQ were determined by comparing the evaluated concentration with the added one.

For the preparation of quality control samples, working solutions of compound 1 (10, 100, and 1,000 μg/ml) were made with methanol in a polystyrene tube. A 100 μl of corresponding working solutions was evaporated to dryness under a nitrogen stream in a polystyrene tube, and then 2 ml of blank plasma was added and mixed to generate 0.5, 5, and 50 μg/ml of quality control samples. Afterwards, 100 μl aliquot of each sample was stored at -20°C in the dark for quality control. The intra-day variation for multiplicate assays (n=5) and the inter-day variation over a four-day period (n=20) were determined using 0.5, 5, and 50 μg/ml of quality control samples. The accuracy and the precision of the assay were determined by measuring the concentration of compound 1 using calibrator and comparing them with the added concentrations. The absolute recovery of compound 1 was determined by comparing peak area of treated quality control sample with that of untreated standard (corresponding standard in the mixture of methanol:water:$ZnSO_4$=1:1:1/2). The stock and working solutions of compound 1 were stable at -20°C in the dark at least for 6 months.

Sample preparation

For the analysis of compound 1 in the plasma of rats and dogs, a deproteinization method was applied using methanol and 10% zinc sulfate. A 100 μl of diluted working solutions was mixed with 100 μl of blank plasma and 50 μl of 10% $ZnSO_4$ for calibrator, whereas a 100 μl aliquot of sample was mixed with 100 μl of methanol and 50 μl of 10% $ZnSO_4$ for quality control samples, study samples, and stability samples (plasma) in a 1.5 ml polypropylene tube. After centrifugation for 20 min at 14,000 g to obtain a clear supernatant, 100 μl of the supernatant was injected directly onto the HPLC column. All procedures for sample handling and processing were carried out at 4°C.

Chromatography

Compound 1 was quantified using a reversed-phase (RP) HPLC on a PLRP-S C_{18} column (150×4.6 mm, 5 μm particle size, Rainin, Emeryville, CA, USA) fitted with a PLRP-S C_{18} Guard-Pak precolumn (Rainin). The HPLC system consisted of Class-LC10A system control software (Shimadzu, Tokyo, Japan), a CBM-10A communication bus module (Shimadzu), two LC-10AD pumps (Shimadzu), a SIL-10A autoinjector with sample cooler (Shimadzu), a RF-10A spectrofluorometric detector (Shimadzu), and a GLP-2050+ laser, Tokyo, Japan printer (LG Elect., Seoul, Korea). The mobile phase was a mixture of 24 parts of acetonitrile and 76 parts of 0.1% TFA in water, and the flow rate was 1 ml/min. The peak area of compound 1 was monitored fluorometrically at an excitation wavelength of 337 nm and an emission wavelength of 460 nm. The retention time of compound 1 was 6.3 min.

RESULTS AND DISCUSSION

The fluorescence response of compound 1 was maximum at an excitation wavelength of 337 nm and an emission wavelength of 460 nm, and was therefore used for the HPLC analysis. Fig. 2 shows typical chromatograms of drug-free rat plasma and dog plasma, drug standards containing 1 μg/ml in plasma, and study samples receiving LB20304a. The deproteinization of samples with methanol and 10% zinc sulfate gave no interferences from endogenous substances in the biological samples. The peak of compound 1 was symmetrical and eluted at 6.3 min at the mobile phase containing acetonitrile and 0.1% TFA. In the preliminary study of the effect of mobile phase on the chromatography of compound 1 having amine and carboxyl groups (Fig. 1), the ion-pairing of amine with 0.1% TFA led to a satisfactory separation with a high theoretical plate, whereas neutral or alkaline buffers resulted in a low theoretical plate and interferences from endogenous substance. The mobile phase containing acetonitrile and buffers of various pHs, however, resulted in a low theoretical plate at a

reversed-phase column having a free silanol group.

The response of detection for compound 1 was linear over the concentration range of 0.2-100 μg/ml in the plasma of rats and dogs based on that all mean back calculated concentrations (evaluated conc.) showed acceptable accuracy (above 85%) and precision (below 15%) (Table I). The mean response factor for each calibrator over the concentration range of 0.2-100 μg/ml did not change over a 4-day period (C.V. was 8.1% for rat and 3.9% for dog). The lower limit of quantification (LOQ) was 0.2 μg/ml using 100 μl of plasma of rats and dogs with a 97-99% accuracy and a 12-14% precision (Table I).

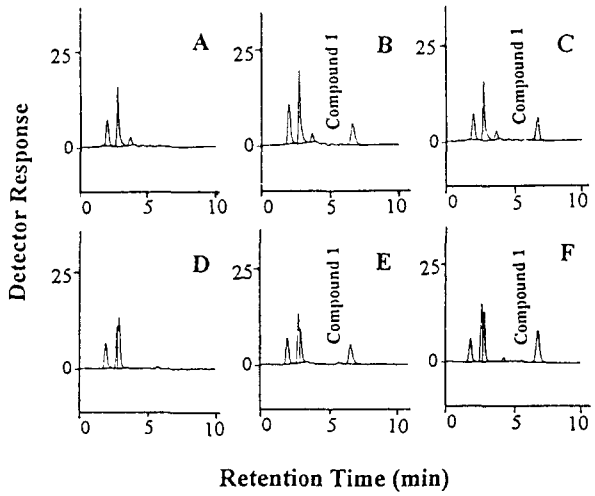

Fig. 2. HPLC chromatograms of (A) drug-free rat plasma, (B) rat plasma spiked with 1 μg/ml of compound 1, (C) plasma obtained from a rat 1 hr after oral administration of LB 20304a at 20 mg/kg, (D) drug-free dog plasma, (E) dog plasma spiked with 1 μg/ml of compound 1, and (F) plasma obtained from a dog 1 hr after oral administration of LB 20304a at 10 mg/kg

The accuracy and the precision of assay for the quality control samples were listed in Table II. The intra- and inter-day accuracy were 88-95% and 88-97%, whereas intra- and inter-day precision (C.V.) were 0.5-6.6% and 0.2-9.3%, respectively, in the plasma of rats and dogs. Under the condition reported here, the absolute recoveries were 68-71% independent of concentration and species.

This assay method was successfully applied to the analysis of compound 1 in the tissue homogenates (Seo *et al.*, 1996). The calibration curve was linear over the concentration range of 0.2 and 100 μg/ml of compound 1 in the liver homogenate samples with the absolute recoveries of 61-71%. The absolute recoveries of compound 1 in the other tissue homogenates at 10 μg/ml were 76% for brain, 51% for submaxillary gland, 35% for thymus, 70% for heart, 59% for lung, 45% for spleen, 56% for stomach, 70% for small intestine, 55% for large intestine, 63% for cecum, 67% for kidney, 70% for testis, 99% for fat, 81% for muscle, and 62% for lymph node samples. However, this method was not applicable to the analysis of compound 1 in the urine and bile samples due to the appearance of endogenous substances. A liquid-liquid extraction method (unpublished data) was applied to the analysis of compound 1 in the urine or bile samples.

The stability of compound 1 was tested in the vari-

Table I. Calibration of the assay of compound 1 determination in the plasma (n=4)

Animal	Added Conc. (μg/ml)	Response factor[a]	Evaluated Conc.[b](μg/ml)	Accuracy[c](%)	Precision[d](%)
Rat	0.2	98248.0 (5586.00)	0.194 (0.0280)	97	14
	0.5	99423.0 (10271.6)	0.488 (0.0570)	98	12
	1	104721 (7078.32)	1.03 (0.0800)	103	7.8
	5	104701 (6015.44)	5.14 (0.280)	103	5.5
	10	107038 (6707.51)	10.5 (0.640)	105	6.1
	50	109165 (5735.61)	53.6 (3.20)	107	6.0
	100	105362 (5190.51)	104 (5.70)	104	5.5
Dog	0.2	100923 (11797.0)	0.197 (0.0240)	99	12
	0.5	97431.0 (7057.44)	0.478 (0.0280)	96	6.0
	1	100988 (6691.57)	0.990 (0.0410)	99	4.1
	5	100050 (4390.76)	4.92 (0.230)	98	4.7
	10	101861 (4700.26)	10.0 (0.450)	100	4.5
	50	104703 (3944.59)	51.5 (2.40)	103	4.7
	100	101759 (4113.66)	100 (5.20)	100	5.2

[a]Peak area divided by its concentration (μg/ml); mean (SD)
[b]Determined from mean response factor; mean (SD)
[c]Evaluated conc. divided by its added conc.; mean
[d]Evaluated conc. divided by its added conc.; C.V.

Table II. Accuracy and precision of the assay for compound 1 determination in the quality control plasma

Animal	Added Conc. (μg/ml)	Evaluated Conc.[a] (μg/ml)		Accuracy[b] (%)		Precision[c] (%)		Recovery[d] (%)	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Mean	C.V.
Rat	0.5	0.44	0.44	88	88	6.1	9.3	68	6.3
	5	4.71	4.79	94	96	1.1	6.6	70	2.4
	50	46.6	47.7	93	95	0.6	7.2	69	2.0
Dog	0.5	0.47	0.47	94	94	6.6	5.6	71	5.6
	5	4.72	4.85	94	97	1.5	1.0	71	1.0
	50	47.5	48.3	95	97	0.5	0.2	69	0.2

[a]Determined from mean response factor; mean of n=5 for intra-day and n=20 for inter-day
[b]Evaluated conc. divided by its added conc.; mean of n=5 for intra-day and n=20 for inter-day
[c]Evaluated conc. divided by its added conc.; C.V. of n=5 for intra-day and n=20 for inter-day
[d]Absolute recovery compared with untreated standard; n=20

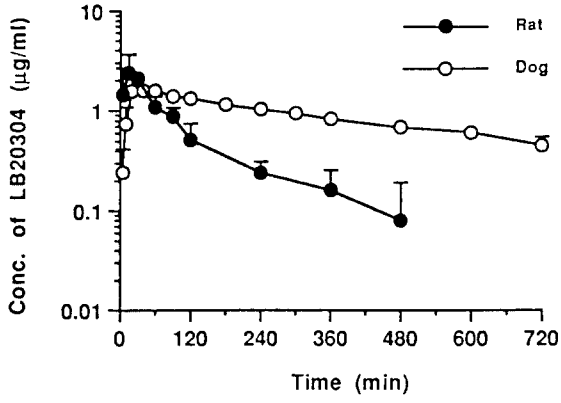

Fig. 3. The concentration-time profiles of compound 1 in the plasma of rats and dogs following oral administration of LB20304a at the dose of 20 mg/kg (rat) or 10 mg/kg (dog) (mean±SD, n=4)

ous specimens. 100 μl aliqouts of compound 1 (2 μg/ml) in methanol, plasma, and 50 mM phosphate buffer of pH 7 (25°C only) were stored at -20, 4, and 25°C in the dark for 1 month. Additionally, compound 1 in 50 mM phosphate buffer of pH 7 was stored at 25°C in the light (400 lux) to examine light sensitivity. Afterwards, triplicates were sampled at determined time schedules and HPLC assayed. The compound 1 was stable in methanol, independent of temperature for 1 month, and stable at -20°C at least for 6 months. In the rat plasma, the compound 1 was stable at -20°C, but 18% of compound 1 disappeared at 25°C for 4 weeks storage. However, compound 1 was not detected in the phosphate buffer of pH 7 at 1 week of light exposure with the many unknown peaks, indicative of a light sensitivity. It is known that a fluoroquinolic structure is light (UV) labile and the degradation products appear to cause phototoxicity (Marutani *et al.*, 1993). Therefore, the storage and HPLC injection of sample was conducted with the prevention of light.

The pharmacokinetics of compound 1 was studied in the SD male rat (200-300 g) and Beagle male dog (8-12 kg). LB20304a was orally administered into 12 hr fasted animals at the dose of 20 mg/4 ml/kg for rat and 10 mg/2 ml/kg for dog via gavage (n=4). Afterwards, approximately 250 μl of blood was collected via the femoral artery of rat or the right cephalic vein of dog up to 720 min, centrifuged for 1 min at 14,000 g, and then 100 μl of plasma was stored in a polypropylene tube at -20°C until assay. The time courses of compound 1 in the plasma following oral administration in rats and dogs are shown in Fig. 3. The peak concentration was 2.2 μg/ml at 18 min for rats and 1.7 μg/ml at 53 min for dogs.

In summary, a simple and reproducible reversed-phase high-performance liquid chromatographic assay method using deproteinization and fluorescence detection was developed for the determination of compound 1 in the plasma of rats and dogs. The extraction recovery was 68-71% and the lower limit of quantification was 0.2 μg/ml in 0.1 ml plasma. Applicability of this method to a pharmacokinetic study was demonstrated. Thus, the above assay method appears to be suitable for the determination of compound 1 in the biological samples for preclinical pharmacokinetics.

REFERENCES CITED

Kim, M. Y., Oh, J. I., Paek, K. S., Hong, C. Y., Kim, I. C. and Kwak, J. H., *In vitro* activities of LB20304, a new fluoroquinolone. *Arch. Pharm. Res.*, 19, 52-59 (1996).

Marutani, K., Matsumoto, M., Otabe, Y., Nagamuta, K., Tanaka, K., Miyoshi, A., Hasegawa, T., Nagano, H., Matsubara, S. and Kamode, R., Reduced phototoxicity of a fluoroquinolone antibacterial agent with a methoxy group at the 8 position in mice irradiated with long-wavelength UV light. *Antimicrob. Agents Chemother.*, 37, 2217-2223 (1993).

Seo, M. K., Lee, S. H., Choi, Y. J., Jeong, Y. N., Lee, S. H., Kim, I. C. and Lee, Y. H., Pharmacokinetics of LB20304, a new fluoroquinolone, in rats and

dogs. *Arch. Pharm. Res.*, in press (1996).
Shah, V. P., Midha, K. L., Dighe, S., McGilveray, I. J., Skelly, J. P., Yacobi, A., Layloff, T., Viswanathan, C. T., Cook, C.E., McDowall, R.D., Pittman, K. A. and Spector, S., Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. *Pharm. Res.*, 9, 588-592 (1992).