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Short Communication

High-performance liquid chromatographic method for the determination of a novel leukotriene D_4 antagonist (MK-0571) in biological specimens

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ABSTRACT

A rapid, sensitive and selective liquid chromatographic procedure was developed to quantitate the levels of a novel leukotriene D_4 antagonist, MK-0571 (I), in biological samples. The method involves the addition of an internal standard, an analogue of I, and methanol to the biological matrix. Following centrifugation the supernatant is chromatographed isocratically on a C_{18} reversed-phase column and the acids are detected with an ultraviolet detector. The sensitivity of the method is such that 50 ng of drug can be quantitated per aliquot of sample. Assays were linear over a 0.06–40.0 μg range and exhibited a recovery of 100.5 ± 7.0% (mean ± S.D.) over this range. This procedure was utilized to monitor plasma, liver and urinary levels of I in chronic and acute toxicity studies in several animal species.

INTRODUCTION

The sulfidopeptide leukotrienes (LTC_4, LTD_4 and LTE_4), which are derived from arachadonic acid, act on respiratory smooth muscle and affect mucocilliary clearance and vascular permeability [1]. These metabolites have been postulated as important mediators in the etiology of human asthma [2–5]. It has been reported [6] that these entities exert their potent pharmacological effects in human lung through the interaction with specific receptors on which LTD_4 exerts the most potent effect.

The interactions of numerous analogues of leukotrienes with the LTD_4 receptor have been investigated [7] in search of an agent to treat asthma. 3-((((3-(2-(7-Chloroquinolin-2-yl)-(*E*)-ethenyl)phenyl)-3-dimethylamino-3-oxopropylthio) methyl)thio)propionic acid (MK-0571, I), has been synthesized and characterized as a potent and selective LTD_4 receptor antagonist in several *in vitro* and *in vivo* model systems [8–11]. This report describes a high-performance liquid chromatographic (HPLC) method that was developed to quantitate levels of I in biolog-

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ical samples from several species. A recent publication describes HPLC methodology for the analysis of I in pharmaceutical preparations [12].

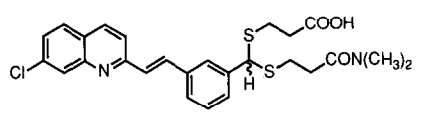
EXPERIMENTAL

Reagents and materials

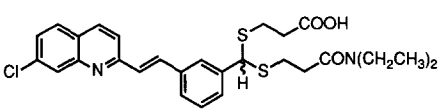
3-((((3-(2-(7-Chloroquinolin-2-yl)-(*E*)-ethenyl)phenyl)-3-dimethylamino-3-oxopropylthio)methyl)thio)propionic acid (MK-0571, I) and its diethyl analogue (II) (Fig. 1), which served as the internal standard (II), were synthesized [1–3] at Merck Frosst Centre for Therapeutic Research (Pointe Claire-Dorval, Canada). These entities were supplied as the sodium salts, however, all concentrations and doses are expressed in terms of the free acids. Stock solutions of I and II were prepared in methanol at a concentration of 1.0 mg/ml, and all subsequent dilutions were made with a mixture of methanol – 0.001 *M* sodium bicarbonate ($NaHCO_3$) (1:1, v/v). Working solutions of I were prepared at the following concentrations: 400, 200, 100, 50, 25, 10, 5.0, 2.5, 1.25 and 0.625 μg/ml and the I.S. was prepared at a concentration of 20 μg/ml.

Other chemicals used include the following: methanol, 85% phosphoric acid and ethyl acetate were all HPLC grade and were purchased from Fisher Scientific (Springfield, NJ, U.S.A.). $NaHCO_3$ was purchased from Sigma (St. Louis, MO, U.S.A.) and HPLC-grade triethylamine (TEA) was obtained from Pierce (Rockford, IL, U.S.A.).

The materials used in this method include the following: 13-ml glass centrifuge tubes (Kontes, Vineland, NJ, U.S.A.), 2-ml amber glass autosampler vials (Sunbrokers, Wilmington, NC, U.S.A.), 100- and 1000-μl adjustable-volume pipetmen (Gilson, Middleton, WI, U.S.A.), Eppendorf repeater pipet (Brinkmann, Westbury, NY, U.S.A.), a linear shaker (Eberbach 6000, Eberbach, Ann Arbor, MI, U.S.A.), a Multivap analytical evaporator (Organomation Assoc., South Berlin, MA, U.S.A.), a Polytron homogenizer (Brinkmann), and a Vortex Genie II (Fisher Scientific).



I



II

Fig. 1. Structures of MK-0571 (I) and internal standard (II).

Assay procedure

Plasma samples were isolated from heparinized whole blood specimens following centrifugation at 15°C. An aliquot of plasma (usually 200 μl) was added to a 13-ml glass centrifuge tube followed by the addition of 100 μl of II solution (2.0 μg), 1 ml of methanol and 100 μl of the methanol–0.001 *M* $NaHCO_3$ solution. When the standards were prepared 100-μl aliquots of the various working standard solutions were added instead of the methanol–$NaHCO_3$ solution. The tubes, which were vortexed at high speed, were then shaken for 10 min. Following centrifugation for 5 min, an aliquot ($\sim$ 800 μl) of the supernatant was transferred to a vial containing an approximately equal volume of 0.005 *M* $NaHCO_3$.

Liver specimens were rinsed in saline, weighed and frozen at −20°C until analysis. Liver samples of known weights were homogenized with 4 volumes of water for approximately 20 s at high speed. An aliquot of the homogenate, usually 300 μl, was added to a 13-ml glass centrifuge tube and combined with 200 μl of II, 300 μl of acetonitrile, and 100 μl of appropriate standard or 0.001 *M* $NaHCO_3$–methanol (1:1) depending on whether it is a standard or sample, respectively. Following the addition of 2 ml of ethyl acetate (vortex-mixed, 0.1 min), the sample was shaken and centrifuged as above. Approximately 1.7 ml of organic solvent were transferred to a clean centrifuge tube and the organic layer was evaporated to dryness by blowing a gentle stream of nitrogen over the sample in a 40°C water bath. The dried residue was dissolved in 800 μl of methanol, vortex-mixed, and an equal volume of 0.005 *M* $NaHCO_3$ was added prior to HPLC analysis. Urine samples were analyzed using a similar procedure.

Apparatus

Analyses were performed on an HPLC system consisting of a Perkin-Elmer (Norwalk, CT, U.S.A.) Series 410 pump with controller and SEC-4 chamber, and an ISS-100 autosampler, a Spectra-Physics (San Jose, CA, U.S.A.) integrator (Model SP-4270), and a Kratos (Applied Biosystems, Ramsey, NJ, U.S.A.) ultraviolet detector (Model 783). Chromatographic separations were achieved using a reversed-phase C_{18} column (Whatman Partisphere, 5 μm particle size, 11.0 cm × 0.47 cm I.D., Whatman, Clifton, NJ, U.S.A.) with a Brownlee (NewGuard RP-18, 7 μm particle size, 1.5 cm × 0.32 cm I.D., Applied Biosystems) precolumn.

Chromatographic conditions

For all plasma samples the separations were performed isocratically at ambient temperature and a flow-rate of 1.0 ml/min. The mobile phase consisted of methanol–0.1 % H_3PO_4 (containing 0.1% TEA) (73:27, v/v). The analytes were monitored at 282 nm and exhibited retention times of about 8 and 12 min for I and II, respectively. The injector and loop were flushed with a methanol–water (70:30) mixture after each injection. A 250-μl aliquot of sample matrix was usually injected at 20-min intervals. When liver and urine samples were analyzed, a

gradient wash step was incorporated after the elution of the analytes to remove endogenous components.

Animal studies

One hundred and twenty male and female non-fasted Sprague–Dawley rats (Charles River Breeding Labs., Wilmington, MA, U.S.A.) were used in a three-month safety evaluation study. The animals were dosed orally with I at various dose levels (200, 400 and 800 mg/kg per day). Heparinized blood specimens ($\sim$ 1 ml) were obtained at 1, 2, 4, 6 and 24 h via the orbital sinus during drug week 8 from three rats/sex/dose groups.

In addition, heparinized blood specimens were obtained from 32 male and female rhesus monkeys (*Macaca mulatta*) following chronic oral dosing for three months with I in the 20–500 mg/kg per day range. Blood samples were taken at 0, 1, 2, 4, 6, and 24 h during weeks 2, 9, and 13 of drug dosing. Plasma samples were isolated as described and frozen until analyzed for drug content. In both studies, liver specimens ($\sim$ 2 g) obtained at necropsy were also analyzed for drug content.

RESULTS AND DISCUSSION

Chromatographic separation

The chromatographic profiles from control and spiked monkey plasma using the aforementioned procedure are shown in Fig. 2. Panel A illustrates a typical control plasma, panel B shows control plasma to which the II was added, panel C illustrates the results obtained when control plasma was spiked with I and II, and panel D presents a typical 1-h plasma profile from an animal dosed orally with 100 mg/kg I per day. As indicated, blank control plasma exhibited no interferences relative to the analytes. Under these conditions I, II, and metabolite (M) exhibited excellent resolution and peak shape with retention times of $\sim$ 9, 13, and 16 min, respectively.

Similar profiles were observed with rat liver homogenates (Fig. 3). Under the conditions of gradient analysis the retention times of I and II were $\sim$ 8.2 and 12.3 min, respectively. As illustrated, a wash step was needed to remove endogenous components. Unlike the monkey, there was no evidence of a metabolite peak with rat plasma.

Assay linearity and accuracy

In most instances, standard recovery data were generated using ten-point calibration curves which were run concurrently with every set of unknown samples analyzed. The relative recoveries were determined by comparing the peak-area ratios (PARs) of the extracted entities I/II to the equation generated by linear regression analysis of the standard curve, having the format $y = mx + b$ where x is the PAR value and y is μg of I. Recovery from monkey plasma using an equation generated from standard data ($n = 120$) was 100.5 $\pm$ 7.0%. The method was

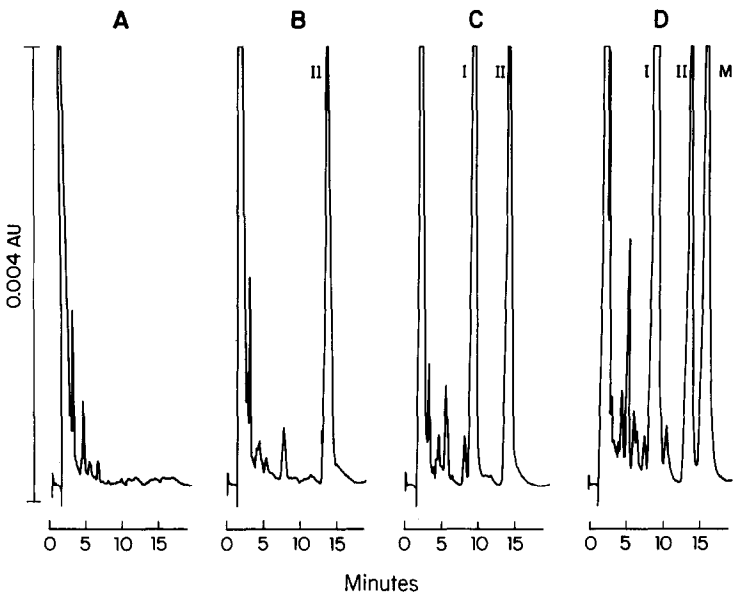


Fig. 2. Chromatographic profiles of (A) control rhesus monkey plasma, (B) control monkey plasma spiked with 2 μg of II, (C) control monkey plasma spiked with 2.5 μg of I and 2 μg of II, and (D) a typical monkey plasma sample taken 1 h after oral dosing with 100 mg/kg/I per day. Peak M is a metabolite of I.

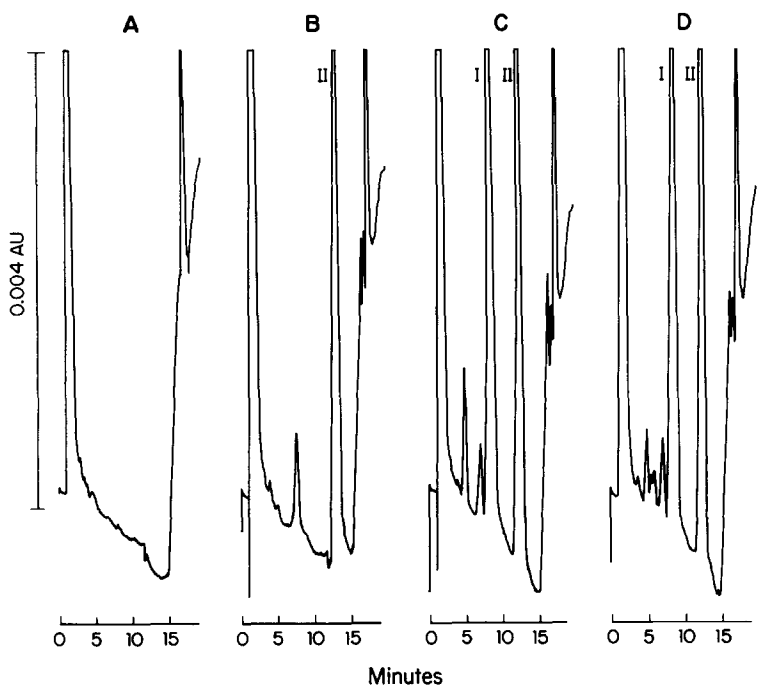


Fig. 3. Chromatographic profiles of (A) control rat liver homogenate, (B) control rat liver homogenate spiked with 4 μg of II, (C) control rat liver homogenate spiked with 2.5 μg of I and 4 μg of II, and (D) a rat liver sample taken at 2 h after oral dosing with 400 mg/kg/I per day.

TABLE I

MEAN RAT PLASMA LEVELS AND AUC VALUES OF I

Values represent the mean of three animals per time period.

Time (h)	Sex	Concentration (μg/ml)		
		800 mg/kg per day	1200 mg/kg per day	1600 mg/kg per day
1	M	63.5	84.1	122.7
	F	130.4	208.3	199.7
2	M	66.2	58.1	126.8
	F	82.0	119.2	180.6
4	M	43.8	42.3	94.8
	F	38.8	93.8	100.9
6	M	37.0	56.2	70.3
	F	58.6	85.2	98.8
24	M	4.9	3.1	13.1
	F	9.7	22.5	22.6
AUC (μg·h/ml)	M	667.0	846.9	1330.2
	F	1009.1	1641.3	1875.3

linear over the entire concentration range (0.0625–40.0 μg per 200 μl) and the detection limit was 0.050 μg per 200 μl of plasma assayed. Similar results were obtained in monkey liver homogenate (102.6 ± 16.0%, $n = 81$). The mean relative recovery (±S.D.) of I for all plasma samples from various studies was 101.0 ± 11.3 ($n = 379$).

Application of method in animal studies

The method has been applied to safety evaluation studies of I in several animal species to establish drug absorption, toxicokinetic parameters, and relative bioavailability at the doses used. Table I illustrates some typical results obtained in rats following oral dosing with 800, 1200, and 1600 mg/kg I per day for eight weeks. In general, the mean peak plasma drug concentrations occurred at 1 h regardless of dose level or sex of animal. Female rats exhibited higher plasma levels (about 1.5-fold) than male rats. These differences are reflected in the total systemic exposure, as assessed by area under the plasma concentration *versus* time curve (AUC), at any given dose. Also of note is the fact that the AUC values increased in a linear dose-proportional fashion for each sex in this dose range and established that linear drug kinetics are applicable in this species.

The HPLC method has also been applied in the toxicokinetic evaluation in rhesus monkeys. Fig. 4 illustrates the results obtained following oral dosing of I to three female rhesus monkeys at doses of 20, 100, and 500 mg/kg per day. The data were generated during drug week 2. These data indicated that the systemic

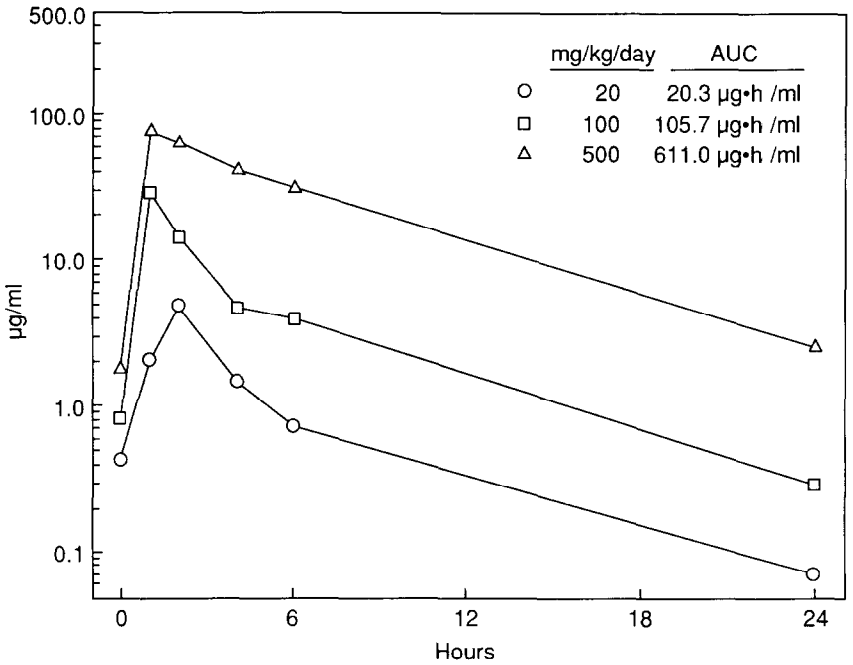


Fig. 4. Plasma concentration of I *versus* time profiles of three rhesus monkeys after oral dosing in the 20–500 mg/kg per day range for two weeks.

exposure (AUC) increased in a linear dose-proportional manner and that the bioavailability was comparable at these dose levels. In addition, the terminal plasma half-lives calculated by linear regression analysis of the log-linear portion of the plasma concentration *versus* time curves were similar (4.3–5.1 h) for these three doses.

CONCLUSION

A precise, accurate, and sensitive HPLC bioanalytical method has been devised for the determination of I in biological specimens. The method has been utilized in the analyses of over 3000 plasma, urine, or liver homogenate samples from rat, rabbit, monkey, mouse, and dog studies used to support the safety evaluation of I. The method is linear over a wide concentration range (0.06–40.0 μg per 200-μl plasma aliquot assayed).

REFERENCES

1 P. J. Piper, *Physiol. Rev.*, 64 (1984) 744.
2 J. M. Drazen, K. F. Austin, R. A. Lewis, D. A. Clark, G. Goto, A. Marfat and E. J. Corey, *Proc. Natl. Acad. Sci.*, 77 (1980) 4354.

3 T. R. Jones, C. Davis and E. E. Daniel, *Can. J. Physiol. Pharmacol.*, 60 (1982) 638.
4 N. C. Barnes, P. J. Piper and J. F. Costello, *J. Allergy Clin. Immunol.*, 79 (1987) 816.
5 S. E. Dahlen, P. Hedqvist, S. Hammerstrom and B. Samuelsson, *Nature (London)*, 288 (1980) 484.
6 C. K. Brucker, R. D. Krell, R. B. Laravuso, B. B. Coursin, P. R. Berstein and J. A. Will, *J. Pharmacol. Exp. Ther.*, 237 (1986) 558.
7 J. Rokach, Y. Guindon, R. N. Young, J. Adams, and J. G. Atkinson, in J. Apsimon (Editor), *The Total Synthesis of Natural Products*, Vol. 7, Wiley-Interscience, New York, 1988, p. 141.
8 R. N. Young, R. Zamboni, M. Belley, E. Champion, L. Charette, R. DeHaven, R. Frenette, A. W. Ford-Hutchinson, J. Y. Gauthier, T. R. Jones, S. Leger, C. S. McFarlane, P. Masson, H. Piechuta, S. S. Pong, J. Rokach and H. Williams, in U. Zor, A. Naor and A. Danon (Editors), *Leukotrienes and Prostanoids in Health and Disease, New Trends in Lipid Mediators Research*, Vol. 3, S. Karger, Basel, 1989, p. 62.
9 R. Zamboni, T. R. Jones, M. Belley, E. Champion, L. Charette, R. DeHaven, R. Frenette, J. Y. Gautheri, S. Leger, P. Masson, C. S. McFarlane, S. S. Pong, H. Piechuta, J. Rokach, M. Therien, H. W. R. Williams and R. N. Young, *J. Med. Chem.*, in press.
10 T. R. Jones, R. Zamboni, M. Belley, E. Champion, L. Charette, A. W. Ford-Hutchinson, R. Frenette, J. Y. Gauthier, S. Leger, P. Masson, C. S. McFarlane, H. Piechuta, J. Rokach, H. Williams, R. N. Young, R. N. DeHaven and S. S. Pong, *Can. J. Physiol. Pharmacol.*, 67 (1989) 17.
11 A. W. Ford-Hutchinson, in B. Samuelsson (Editor), *Advances in Prostaglandin, Thromboxane and Leukotriene Research*, Raven Press, New York, 1989, p. 507.
12 P. R. Bedard and M. L. Cotton, *J. Liq. Chromatogr.*, 12 (1989) 307.