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Short Communication

Determination of (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid), a novel leukotriene biosynthesis inhibitor, in human plasma and urine by liquid chromatography

W.F. KLINE, Y.-H.R. HAN, B.K. MATUSZEWSKI* and W.F. BAYNE

Merck Sharp & Dohme Research Laboratories, West Point, PA 19486, USA

Keywords: *Plasma and urine; leukotriene biosynthesis inhibitor — HPLC assay; ultraviolet detection; anion-exchange solid-phase extraction; diamino extraction cartridge.*

Introduction

Compound **I**, (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid) (Fig. 1), is a potent inhibitor of leukotriene biosynthesis both *in vitro* and *in vivo* [1, 2]. In order to support human pharmacokinetic studies employing 100–750 mg oral doses, an assay in plasma and urine with the limit of reliable quantification (LQ) of 25 ng ml^{-1} was necessary and has been developed. The assay utilizes the combined liquid–liquid and solid phase extraction of **I** from plasma and urine, followed by liquid chromatography (LC) with ultraviolet (UV) detection. The assay described in this paper has been successfully applied in clinical studies.

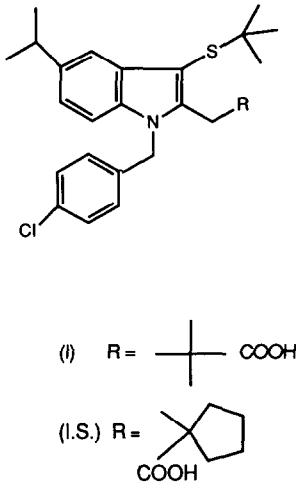

Figure 1
Chemical structure of **I** and the internal standard (I.S.).

Experimental

Reagents and chemicals

HPLC-grade acetonitrile, ethyl acetate, isopropyl alcohol, toluene and analytical-grade sodium mono- and di-hydrogen phosphate, glacial acetic acid, phosphoric acid, and the Glusulase reagent containing 10,000 units of sulfatase and 90,000 units of glucuronidase were from Fisher (Fair Lawn, NJ, USA). Methyl-*t*-butyl ether was obtained from Burdick & Jackson Laboratories (Muskegon, MI, USA), and tetrabutylammonium hexafluorophosphate (TBAHFP) from Fluka (Ronkonkoma, NY, USA). Compound **I** and the internal standard (I.S.) were obtained from Merck Sharp and Dohme Research Laboratories (Rahway, NJ, USA). Frozen heparinized control plasma was supplied by Biological Specialties Co. (Lansdale, PA, USA). Deionized water was prepared using a Milli-Q Reagent Water System (Millipore, Milford, MA, USA). A solution of 0.2 M monohydrogen phosphate in water adjusted to pH 3 with concentrated phosphoric acid (solution A) was

* Author to whom correspondence should be addressed.

utilized for lowering the pH of plasma and urine before extraction. A mixture of toluene–ethyl acetate–isopropyl alcohol (50:49:1, v/v/v) was used as an extracting solvent (solvent B).

Instrumentation

A Varian 9010 solvent delivery system with a Varian 9095 autosampler (San Fernando, CA, USA) and a Kratos (Ramsey, NJ, USA) model 773 UV detector interfaced to a Hewlett-Packard (Palo Alto, CA, USA) 3357 laboratory automation system (LAS) were used for all analyses. Absorption and fluorescence spectra were obtained using a diode array spectrophotometer (HP 8452A) and a Perkin–Elmer spectrofluorometer (Model 650-10S, Norwalk, CT, USA). Absorption spectra were also monitored directly from LC runs using a photodiode array UV detector (Polychrom 9060, Varian). An LC-17A electrochemical (EC) flow cell with a dual glassy carbon working electrode and an LC-4B amperometric controller (Bioanalytical Systems, West Lafayette, IN, USA) were utilized in the exploratory stages of assay development when EC detection was evaluated. Cyclic voltammetry measurements were performed using a Bioanalytical Systems model CV-1B cyclic voltammograph.

The analytical column was a Beckman (Fullerton, CA, USA) Ultrasphere RP-8 (5 μm, 250 mm × 4.6 mm) column. A 1.5 cm × 0.32 cm Applied Biosystems (Foster City, CA, USA) New-Guard RP-8 (7 μm) column was utilized as a guard column. For solid phase extraction (SPE), Baker (Phillipsburg, NJ, USA) Bakerbond diamino (NH/NH2) cartridges (3 ml) were used. These cartridges were attached to an Applied Separations (Bethlehem, PA, USA) SPE-30 extraction manifold. The centrifuge was a Model K, size 2 unit made by International Equipment Company (Needham Heights, MA, USA).

Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile and 0.015 M phosphoric acid in water (90:10, v/v). The mobile phase was filtered through a 0.2 μm Nylon 66 filter (Rainin Instruments, Woburn, MA, USA). The column was maintained at room temperature at a flow rate of 1 ml min^{-1}. The UV detector was set at 290 nm and a sensitivity of 0.001 AUFS.

Standard solutions, calibration and quality control samples

Five working standard solutions of **I** (0.5, 1.0, 2.0, 4.0, 7.0 and 10.0 μg ml^{-1}), and one working standard solution of the I.S. (4 μg ml^{-1}) were all prepared in acetonitrile by the appropriate dilution of stock standards. Additional stock standards, independent from those used for preparing working standards, were used to make quality control (QC) samples. The QC samples in plasma were prepared in 1.25 ml aliquots and stored at −20°C; after thawing, 1 ml aliquots were assayed. The urine QC samples were stored as 1 ml aliquots and the whole contents of each tube were used for the analysis.

A six-point calibration curve was run daily by spiking 1 ml of control biological fluid with 50 μl of the appropriate working standard/acetonitrile mixture and 50 μl of the I.S. working solution. In addition, a set of QC standards at 50 and 400 ng ml^{-1} was analysed with each day's study samples.

Sample preparation

The frozen plasma or urine samples were thawed to room temperature and vortexed for 10 s. To a 1 ml aliquot of biological fluid placed in a 16 × 125 mm borosilicate glass centrifuge tube, 50 μl of the working I.S. solution, 1 ml of solution A and 2 ml (plasma) or 3 ml (urine) of acetonitrile were added and the mixture was vortexed for 60 s. The larger volume of acetonitrile used for the urine extraction was necessary to improve the recovery of **I**. Urine samples were kept for 30 min at 37°C in a water bath to facilitate the dissolution and extraction of **I** and the I.S. Both compounds were extracted with 5 ml of extracting solvent B, vortexed for 2 min, and centrifuged at 5300*g* and 10 min. The top organic layer (6 ml) was transferred to a 13 × 100 mm culture tube and applied, in three portions, to a diamino SPE cartridge preconditioned with 6 ml of solvent B. After washing the cartridge with 3 ml of the same solvent, it was dried under vacuum for 2 min and eluted with 3 ml of 10% acetic acid in methyl-*t*-butyl ether. One millilitre of water was added to the eluent and the samples were vortex-mixed for 2 min and centrifuged for 5 min at 5300*g* to remove excess acetic acid. The top ether layer was then transferred to a 10 × 75 mm culture tube and the solvent was evaporated under a gentle stream of nitrogen at 40°C. The residue

was reconstituted in 240 μl acetonitrile, vortex-mixed for 1 min and sonicated for 30 s. In order to adjust the solvent strength to that of the mobile phase, 60 μl of 0.015 M phosphoric acid in water was added. The samples were vortex-mixed for 1 min and 120 μl of the solution was injected into the LC system.

The presence of **I** in urine in the glucuronide form was assessed by incubating selected patients' urine (1 ml) with 50 μl of Glusulase reagent for 24 h at 37°C, before subjecting the samples to the extraction procedure described previously.

Precision, accuracy, recovery, specificity and linearity

The precision of the method was determined by replicate analyses ($n = 5$) of human plasma and urine at concentrations of 25, 50, 100, 200, 300, 400 and 500 ng ml^{-1}. The linearity of each standard line was confirmed by plotting peak height ratios (I/I.S.) versus the concentration of **I**. Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted linear regression of the standard curve. The weighing factor was the inverse of the variance at each concentration. The accuracy of the assay, expressed as (mean observed concentration/expected concentration) ×100, was determined from the daily analyses of the QC samples. Recovery was calculated by comparison of the peak areas of **I** recovered from plasma with the peak areas of the injected standards. The specificity of the assay was checked by running plasma and urine samples from different sources and from subjects' pre-dose biological fluids; endogenous interferences were not observed.

Results and Discussion

Selection of the detection technique

Several approaches for the detection of **I** after LC separation were initially considered. In addition to UV absorption, fluorescence and electrochemical (EC) detection were explored. Compound **I** was virtually non-fluorescent in the 1:1 (v/v) mixture of acetonitrile and sodium dihydrogen phosphate (0.0125 M, pH 3). Cyclic voltammetry measurements indicated that **I** was EC active. Three oxidation peaks were observed at +0.8, +1.2 and +1.4 V in purely organic solvent (acetonitrile containing 0.1 M of TBAHFP as a supporting electrolyte), and at +0.8, +1.1 and +1.2 V in a mixture of phosphate buffer (0.1 M, pH 7)–acetonitrile (1:1, v/v). The EC reaction was fully reversible. However, the sensitivity of detection using LC with the EC detector set at +0.8 to +1.0 V and with phosphate buffer (0.0125 M, pH 7)–acetonitrile (1:1, v/v) mobile phase was much poorer than with UV detection. Therefore, the method based on UV detection was chosen for the assay.

The UV absorption spectrum of **I** in methanol indicated the presence of two absorption maxima at 226 and 290 nm with molar absorption coefficients (ε) of 40,000 and 13,000 M^{-1} cm^{-1}, respectively. This high UV absorption of **I** allowed for the development of a sensitive assay in biological fluids in the low ng ml^{-1} range. Because the selectivity of detection was much better at 290 nm than at 226 nm, the former wavelength was chosen for the assay.

Extraction and chromatography

Several different approaches to the isolation of **I** from plasma and urine have been studied. Direct liquid–liquid extraction at pH 3 using solvents ranging in polarity from hexane to ethyl acetate produced extracts containing several major interferences. When applied from water, compound **I** was highly retained on a variety of solid phase extraction cartridges including C2, C8, C18 and CN phases, and was quantitatively eluted with ethyl acetate or a mixture of methylene chloride–petroleum ether (1:1, v/v). However, when **I** was applied from plasma or urine under the same conditions, recoveries were poor and a number of impurities coeluted with **I** or the I.S. Because of the highly non-polar character and poor solubility of **I** in organic solvents and because of its high protein binding, a protein precipitation step followed by liquid–liquid and solid phase extraction (SPE) was necessary to achieve the desired detection sensitivity. After protein precipitation with acetonitrile, liquid–liquid extraction of the acidified plasma with solvent B followed by SPE on a weak anion-exchange sorbent (diamino cartridge) gave chromatographically clean extracts with high recoveries. The final elution from the cartridge was accomplished using a mixture of 10% acetic acid in methyl-*t*-butyl ether. The efficient removal of many non-polar impurities was achieved by applying the organic extract directly to the anion-exchange SPE cartridge,

and by additional cartridge washing with highly non-polar solvent B. Additionally, a time-consuming solvent evaporation step was avoided.

The extraction procedure from plasma and urine was essentially the same. However, when urine samples spiked with **I** were stored at −20°C in both plastic and glass containers, the recovery of **I** was poor, probably due to the limited solubility of **I** and its precipitation out of the solution. Therefore, in the clinics, 1 ml aliquots of the urine samples were placed in separate glass tubes *before* freezing; after thawing, the initial liquid–liquid extraction was performed in the same tubes at 37°C for 0.5 h. Under these conditions, the recoveries of **I** and the I.S. in urine were adequate for the assay.

Representative chromatograms of the plasma and urine matrices and various spiked and subjects' samples containing **I** and the I.S. are presented in Figs 2 and 3. The analyses of plasma and urine control samples and various pre-dose samples from subjects indicated the absence of interfering endogenous compounds, confirming the adequate assay specificity.

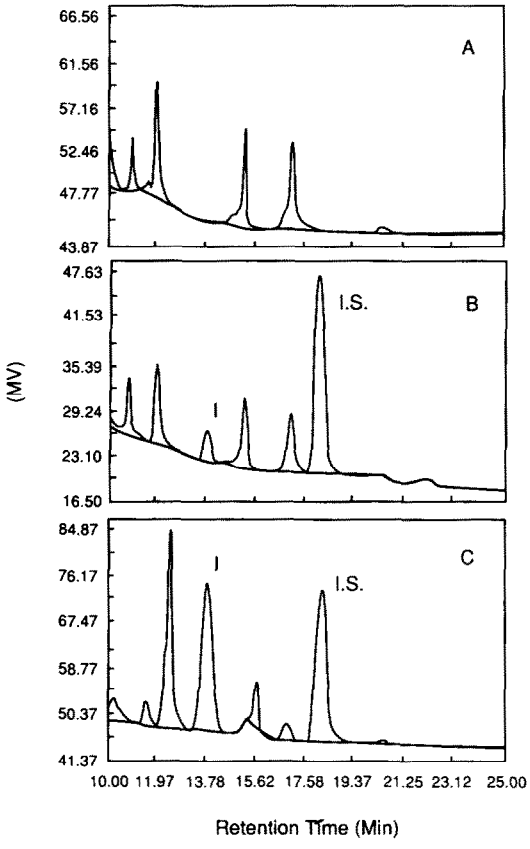

Figure 2
Representative chromatograms of **I** in human plasma. (A) Control plasma. (B) Control plasma spiked with 25 ng ml^{-1} of **I** and 200 ng ml^{-1} of the I.S. (C) Post-dose plasma sample from human subject 1 h after oral dosing with 100 mg of **I**; the concentrations of **I** and the I.S. are equivalent to 230 and 200 ng ml^{-1}, respectively.

Assay validation

The assay was linear in the concentration range 25–500 ng ml^{-1} and typical equations of the linear regression lines were $y = 0.006242x + 0.02557$ and $y = 0.006038x + 0.01471$ in

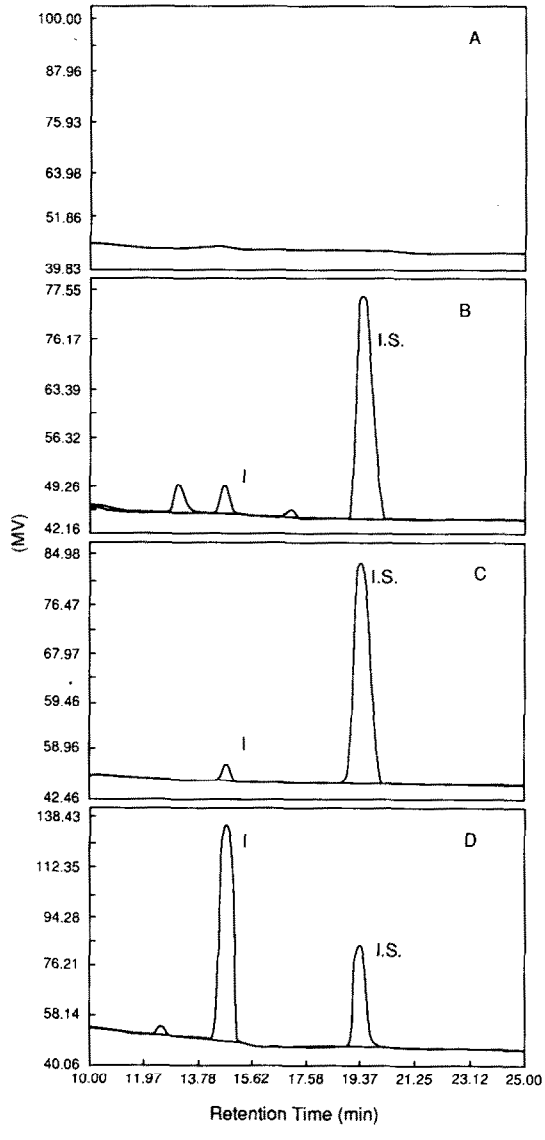

Figure 3
Representative chromatograms of **I** in human urine. (A) Control urine. (B) Control urine spiked with 25 ng ml^{-1} of **I** and 200 ng ml^{-1} of the I.S. (C) Post-dose urine sample of human subject (4–12 h collection interval), after oral dosing with 250 mg of **I** and prior to incubation with Glusulase; the concentrations of **I** and the I.S. are equivalent to <25 and 200 ng ml^{-1}, respectively. (D) Urine sample as in Fig. 3C, but after 24 h incubation with Glusulase; the concentrations of **I** and the I.S. are equivalent to 381 and 200 ng ml^{-1}, respectively.

plasma and urine, respectively. The concentrations calculated from these representative standard curves were within ±6.6% (plasma) and ±2.2% (urine) of the spiked samples throughout this concentration range. The corresponding correlation coefficients of the regression lines were 0.9975 and 0.9981. The within-day precision of the assay was less than 10% (RSD) for all concentrations within the standard curve range (Table 1). Inter-day precision, as measured by the concentration of QC samples, was also below 10% (RSD) (Table 2). The accuracy of the assay was 98–104% (Table 2).

The data in Table 2 also confirm the adequate stability of **I** under storage conditions of −20°C for a period of 5 and 2 weeks in plasma and urine, respectively. In a separate plasma study, a new set of Q.C. samples was analysed and **I** was found to be stable for a period of at least 2 months.

The limit of detection of the assay at a signal-to-noise ratio of 3 was about 5 ng ml^{-1}. Only about 28% of **I** extracted from plasma and about 24% of **I** extracted from urine were actually injected on the column. The limit of reliable quantification, defined as the lowest point on the standard line for which the relative standard deviation (RSD) is ≤10%, was 25 ng ml^{-1}.

Table 1
Within-day precision for the analysis of **I** in plasma and urine; relative standard deviation (RSD) of replicate analyses (n = 5)

Concentration (ng ml^{-1})	RSD (%) Plasma	Urine
25	6.7	4.4
50	4.4	1.6
100	2.8	1.4
200	1.3	1.3
300	5.2	1.1
400	0.7	1.1
500	1.4	1.3

Table 2
Inter-day variability and accuracy data for the assay of quality control plasma and urine samples spiked with **I**

	Plasma (n = 6)*			Urine (n = 3)†		
Spiked concentration (ng ml^{-1})	Mean (ng ml^{-1})	RSD (%)	Accuracy‡ (%)	Mean (ng ml^{-1})	RSD (%)	Accuracy‡ (%)
50	50.1	5.5	100.2	51.8	0.8	103.6
400	406.0	5.4	101.5	394.0	2.0	98.5

*Measured on six different days (n = 6) over a period of 5 weeks.
†Measured on three different days (n = 3) over a period of 2 weeks.
‡Expressed as (mean observed concentration)/(spiked concentration) ×100.

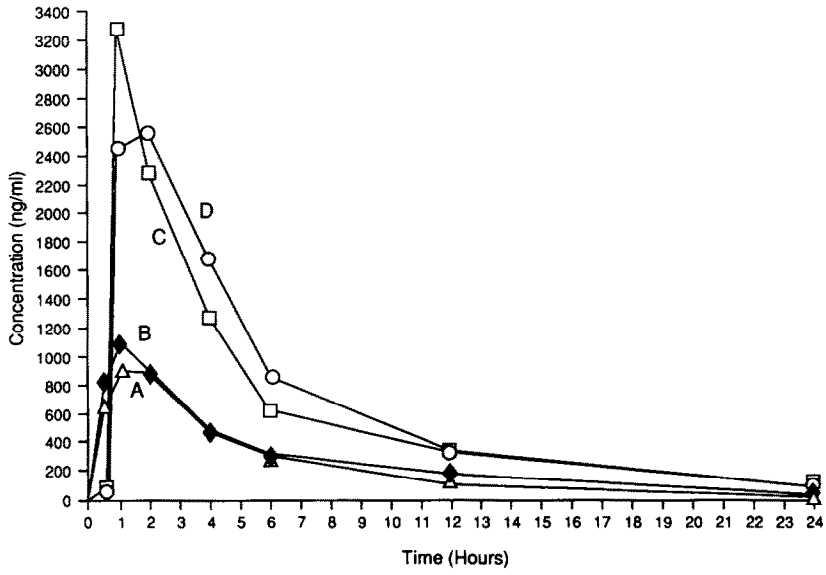

Figure 4
Representative plasma-concentration time profiles of **I** following single oral doses of 100 mg (Subjects A and B) and 250 mg (Subjects C and D) of **I**.

The recovery of **I** from plasma, within the whole assay range, was better than 90%. Similar recovery was observed in urine, as indicated by essentially the same values of the slopes of the standard lines measured from both plasma and urine.

Analysis of samples from clinical studies

The assay was used for the determination of **I** in plasma and urine from patients enrolled in various clinical studies. The representative plasma data for selected subjects dosed with 100 and 250 mg of **I** are presented in Fig. 4.

Measurable levels (>25 ng ml^{-1}) of free, unchanged **I** were not detected in the urine of subjects receiving the 250 mg dose. In order to assess the presence of **I** in the glucuronide form, an incubation step with Glusulase was required. After enzyme incubation, **I** was detected in urine; it was present, however, in extremely small amounts, representing less than 0.1% of the administered dose.

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