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Stereoselective determination of *R*(−)- and *S*(+)-MK-571, a leukotriene D_4 antagonist, in human plasma by chiral high-performance liquid chromatography

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ABSTRACT

A stereoselective high-performance liquid chromatographic method that utilizes fluorescence detection was developed for the selective and sensitive quantification of *R*(−)- and *S*(+)-enantiomers of MK-571 (**1**), a potent and specific leukotriene D_4 antagonist, in human plasma. Racemic **1** was isolated from the acidified plasma using solid-phase extraction and the resulting residue was successfully reacted with isobutyl chloroformate and *R*(+)-1-(1-naphthyl)ethylamine in triethylamine–acetonitrile medium to form the diastereomer of each enantiomer. A structural analogue of **1** was used as internal standard. The derivatized sample was dissolved in 1,1,2-trichlorotrifluoroethane and an aliquot was chromatographed on a (*R*)-urea chiral column using a mobile phase containing 89% triethylamine–pentane (3:1000, v/v), 10% 2-propanol, and 1% acetonitrile at a flow-rate of 1.5 ml/min. The fluorescence response (excitation wavelength, 350 nm; emission wavelength, 410 nm) was linear ($r^2 > 0.999$) for concentrations of enantiomers of **1** from 0.05 μg/ml, the lowest quantitation limit, up to 2.5 μg/ml. Intra-day coefficients of variation at 0.05 μg/ml were 2.4% for the *R*(−)-isomer and 2.0% for *S*(+)-isomer. The corresponding inter-day coefficients of variation for *R*(−)- and *S*(+)-**1** were 2.6 and 3.6%, respectively. The utilit of the methodology was established by analysis of plasma samples from male volunteers receiving single intravenous and oral doses of racemic **1**.

INTRODUCTION

Sodium (±)-3-{[(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)((3-dimethylamino)-3-oxopropyl)methyl]thio}propanoate [MK-571, (±)-**1**, Fig. 1] is a specific and potent leukotriene D_4 (LTD_4) antagonist and has shown *in vitro* and *in vivo* activity in several diverse animal models [1]. The compound, which is currently under development for the treatment of asthma, possesses a chiral center at the methine carbon of the dithio side-chain and exists in two enantiomeric forms. Studies have shown that enantiomers of many drugs possessing chiral center have different pharmacological activities [2], plasma disposition kinetics [3], or metabolism [4]. The two enantiomers of (±)-**1**, without exception, have different plasma binding and clearance rates in rats, dogs, and monkeys [5]. Results from the animal studies indicate the need for developing a stereoselective assay for the

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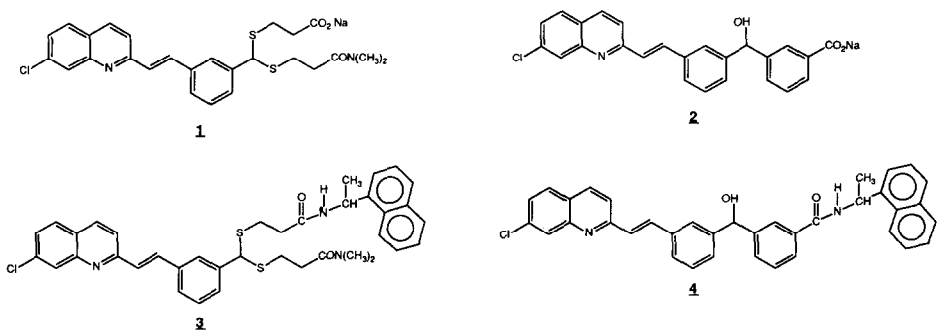

Fig. 1. Structures of (±)-**1**, internal standard (I.S., **2**), and their respective NEA derivatives (**3** and **4**).

determination of the enantiomeric composition of (±)-**1** in biological fluids. Published high-performance liquid chromatographic (HPLC) methods concentrate mainly on three methods for resolving the enantiomeric pairs: the addition of chiral additives to mobile phase [6], the separation of the enantiomers on chiral stationary phases (CSP) [7,8], and the derivatization of the enantiomers to their diastereomers followed by reversed-phase or normal-phase separation [9].

The approach taken in this work has been to prepare the diastereomeric amides of (±)-**1** followed by separation of the amides on a CSP column. In combination with a solid-phase extraction method, the method has been applied to the assay of plasma samples obtained from human subjects administered (±)-**1** intravenously and orally.

EXPERIMENTAL

Chromatographic solvents (hexane, pentane, methanol, acetonitrile, and isopropanol) were obtained from three different vendors (Fisher Scientific, Pittsburgh, PA, USA; Burdick & Jackson, Muskegon, MI, USA; Mallinckrodt, St. Louis, MO, USA). 1,1,2-Trichlorotrifluoroethane (TCTF) was supplied by Burdick & Jackson. Triethylamine (TEA) and *R*(+)-1-(1-naphthyl)ethylamine (NEA) were obtained from Aldrich (Milwaukee, WI, USA) and used without further purification. Isobutyl chloroformate (IBCF) was purchased from Eastman Kodak (Rochester, NY, USA). Racemic (±)-**1** and internal standard (I.S., **2**) were obtained from Merck-Frosst Canada (Quebec, Canada). Standards were prepared in deionized and distilled water with 10 μl of TEA. A SPE21 vacuum manifold with 500-mg octadecasilane-bonded silica (C_{18}) extraction cartridges (J. T. Baker, Phillipsburg, NJ, USA) was used for sample extraction. Fisher screening columns were used for sample filtration and polypropylene tubes with caps (Sarstedt, Princeton, NJ, USA) were used for sample preparation. To minimize the exposure of (±)-**1** to in-house light effects after sample preparation, amber autosampler vials (A. A. Pesce, Kennett Square, PA, USA) were used for injection.

Apparatus

The isocratic separation was performed using a liquid chromatographic system consisting of a Perkin-Elmer Series 4 LC (Norwalk, CT, USA), a PE ISS-100 autosampler and a PE Model 650-10S fluorescence spectrophotometer (excitation wavelength, 350 nm; emission wavelength, 410 nm; slit width, 12 nm). Detector noise was filtered using a Model 1021A filter–amplifier (AnSpec, Ann Arbor, MI, USA) inserted between the detector and a Model 4270 integrator (Spectra Physics, San Jose, CA, USA). Both the 4270 integrator and an HP 3537 laboratory automated system (Hewlett-Packard, Palo Alto, CA, USA) were used for data collection and peak integration. A Supelco LC-Si (5 μm, 50 mm × 4.6 mm I.D.) column was attached in front of a Supelcosil (*R*)-phenylurea analytical column (Supelco, Bellefonte, PA, USA; 5 μm, 250 mm × 4.6 mm I.D.). The analytical column was thermocontrolled at 40°C using a temperature controller (Sys-tech, Minneapolis, MN, USA). The mobile phase [89% triethylamine–pentane (3:1000, v/v), 10% isopropanol, and 1% acetonitrile) was delivered at a flow-rate of 1.5 ml/min with a column back-pressure of 2.5 MPa. A mixture of 20% isopropanol and 80% hexane was used to flush the injection needle. Several chiral stationary phase columns [*R*-N-(3,5-dinitrobenzoyl)phenylglycine (J. T. Baker), β- and γ-cyclodextrin (Advanced Separation Technologies, Whippany, NJ, USA), bovine serum albumin (Macherey Nagel, Düren, Germany), α-acid glycoprotein (Advanced Separation Technologies), cellulose esters (Daicel Chemical Industries, Tokyo, Japan)] were used to evaluate the direct resolution of (±)-**1** enantiomers.

Clinical samples

Plasma samples were collected from healthy male volunteers who received oral or intravenous doses of (±)-**1**. Blood samples were collected at time intervals as specified in the protocol. After separation, plasma samples were stored at −15°C until the time of analysis. At all times during preparation, precautions were taken to minimize the exposure of the specimen to light.

Sample preparation and extraction of plasma

Working standards were prepared at concentrations of 1.0, 2.0, 5.0, 10.0, 25.0, 50.0 and 100.0 μg/ml (±)-**1** in plasma. Samples for standard curves were prepared by adding 0.1 ml of the appropriate working standard, 1.25 μg of I.S. and 0.5 ml of glacial acetic acid to 0.9 ml of human control plasma (Sera-Tec Biologics, New Brunswick, NJ, USA).

Quality control samples were prepared to represent the high and low ends of the standard curve. Plasma was spiked with (±)-**1** at 0.5 and 2.5 μg/ml concentrations; 1-ml aliquots were stored at −15°C. The quality control samples were assayed on each analysis day to ensure the reliability of the system.

Plasma specimens were prepared for extraction by placing 1.0 ml of plasma, 0.05 ml of glacial acetic acid and 0.1 ml of working I.S. (12.5 μg/ml) in a 75 mm ×

11 mm polypropylene tube. After vortex-mixing for 30 s, the plasma sample was applied to a 500-mg Baker C_{18} cartridge, pre-washed with 3 ml of methanol followed by 6 ml of water. The cartridge was further washed with 3 ml of water. Solvents were removed from the cartridge using the house vacuum. The cartridges was then dried by passing 140 Pa of air through the cartridge for 10 min. Methanol (2 × 0.75 ml) was then passed through the cartridge with the eluates being collected in a 75 mm × 11 mm polypropylene tube. The methanol was evaporated, under a nitrogen gas stream, in a 35°C water bath. TEA–acetonitrile reagent (100 μl; 80 mg TEA–10 ml acetonitrile) was added and the mixture was vortex-mixed for 10 s. This was followed by adding 0.1 ml of IBCF–acetonitrile reagent (170 mg IBCF–10 ml acetonitrile). After vortex-mixing for 30 s, the mixture was incubated at room temperature for 4 min. After the final addition of 0.1 ml of *R*(+)-NEA–acetonitrile derivatizing reagent [90 μl *R*(+)-NEA–1 ml acetonitrile, prepared fresh daily], the mixture was vortex-mixed for 1 min and allowed to incubate for 10 min at room temperature. The organic solution was then evaporated under a nitrogen gas stream and the residue was reconstituted with 1 ml of TCTF. A screening column was used to filter a clear solution into an amber autosampler vial. A 50-μl volume of the filtered solution was injected on the HPLC column for analysis. Unknown concentrations of (+)-**1** and (−)-**1** in plasma were calculated from the linear regression equation of the daily standard curve constructed by plotting the peak-height ratios of (+)- and (−)-**1** to I.S. against the concentration of each enantiomer. Samples whose concentrations exceeded the range of the standard curves were diluted and assayed again.

RESULTS AND DISCUSSION

Direct separation of (±)-**1** and its methyl esters were attempted with no success using the chiral stationary phase (CSP) columns described in the Experimental section.

Better separation was reported on CSP columns for amide derivatives of several compounds when compared with the chromatography of their ester counterparts [10]. *R*(+)-α-Methylbenzyl amide (MBA) and *R*(+)-NEA amide derivatives of **1** were prepared according to the procedure described by Vaughan and Osato [11]. Results from the mass spectral analysis of both amide derivatives of **1** confirmed their structures. Several attempts to separate the diastereomers of both amide derivatives of **1** using non-chiral reversed- or normal-phase conditions were unsuccessful despite examples of diastereomeric separation of many compounds in the literature [12,13]. This was probably due to the significant distance of the two chiral centers in the diastereomeric derivatives. These centers are separated, by six bonds, from each other. Such increased distance makes separation of the diastereomeric derivatives difficult [9]. The MBA and NEA derivatives of (±)-**1** were not separable on any of the CSP columns listed in the Experimental section except for the phenylurea column where the MBA derivatives were barely

separated with $\alpha = 1.03$, $k'_1 = 5.50$ and $k'_2 = 5.69$ and the NEA derivatives had much better resolution. It quickly became apparent that forming the amide using a chiral derivatizing reagent, as well as the use of a CSP column, was necessary for enantiomer separation.

Since the NEA derivative–phenylurea column combination looked the most promising, increasing peak resolution was attempted. Various normal-phase constituents were evaluated including chloroform, acetonitrile, ethyl *tert.*-butyl ether, isopropanol, hexane, pentane, and heptane. The best resolution between **1** enantiomer peaks and I.S. was accomplished using 85% TFA–hexane (3:1000, v/v), 10% chloroform, and 5% isopropanol.

Over forty compounds were tested as internal standard candidates. None of the achiral compounds tested showed resolution which would be useful for this assay. The compound **2** showed the most compatible chromatographic characteristics. Although it is a chiral compound and a diastereomeric mixture is formed after derivatization with NEA, no separation between the diastereomers of **4** (Fig. 1) were observed under the described chromatographic conditions. Therefore, this compound was selected as an internal standard.

Some months after optimization, a new phenylurea column was obtained which had radically different characteristics. A change in silica vendors was determined as the cause of the differences. The peak elution order for this new column switched from the original (−)-**1**, (+)-**1**, I.S. to I.S., (−)-**1**, (+)-**1**. Consequently, more work had to be done to optimize resolution and two major changes resulted. First, the mobile phase was re-optimized to 89% TEA–pentane (3:1000, v/v), 10% isopropanol, and 1% acetonitrile. Because of the elimination of the halogenated solvent (chloroform) from the mobile phase, this change was actually quite beneficial from a waste handling standpoint.

After running several hundred samples, it was discovered that the separation between the I.S. and (−)-**1** slowly decreased causing poor quantitation. To correct this problem, the second modification was made which was the insertion of a 5-cm silica column before the analytical column. This had no effect on the separation of the enantiomers but it did increase the separation between I.S. and (−)-**1** peaks. Decrease in peak resolution still occurred, but the silica column prevented any detrimental interference.

During the derivatization optimization, acetonitrile and IBCF were finally chosen as the reaction medium and coupling reagent, respectively, because of better reaction yield and faster reaction rate. Both chiral forms of NEA [*S*(-) and *R*(+)] were tried with both working equally well. *R*(+)-NEA was arbitrarily chosen for this reaction. Elution order was established by chromatographing the NEA derivative of each enantiomer. As expected, the elution order using *S*(−)-NEA was reversed from that found using *R*(+)-NEA.

Liquid–liquid and liquid–solid extraction were both evaluated for plasma isolation and clean-up. Solvents tried for liquid–liquid extraction included ethyl *tert.*-butyl ether, chloroform, ethyl acetate, diethyl ether, and mixtures of hexane

and isopropanol. Limited success was achieved with low recovery being seen. Improved recovery and cleaner samples were obtained using a solid-phase extraction method. Cartridges of octylsilane (C_8) and octadecylsilane (C_{18}) of 1 ml (100 mg) and 3 ml (500 mg) were evaluated. C_{18} cartridges performed the best with 1-ml and 3-ml cartridges giving about the same recovery. The recovery for lower concentrations appeared to be slightly higher using the 3-ml cartridges so these were chosen over the 1-ml cartridges. Also, the 3-ml cartridges were more convenient for sample preparation because of the sample volume involved (> 1 ml). Overall, recovery for **1** and I.S. at the 0.5 and 2.5 μg/ml range was around 80%.

A non-stereoselective assay that measured the total amount of **1** in plasma was also developed and used for the recovery studies. Similar to the stereoselective method, it was based on reversed-phase HPLC with fluorescence detection. An I.S. was added to the plasma samples prior to protein precipitation. A diethylamino analogue of **1** was chosen as the I.S. Acetonitrile in plasma (2:1) was used for protein precipitation. After centrifuging the sample, the resulting supernatant was injected on a column packed with octadecylsilane-bonded silica (DyChrom, Sunnyvale, CA, USA; 250 mm × 4.6 mm I.D., 5 μm). A mobile phase of 16% ammonium phosphate buffer [0.05 *M* $(NH_4)_2HPO_4$, 0.01 *M* H_3PO_4, pH 2.5] and 84% methanol with a flow-rate of 1.5 ml/min was used for peak separation. The same fluorescence detector conditions were used as for the stereoselective assay for quantitation. Retention times for **1** and the I.S. were 3.5 and 4.7 min, respectively. This non-stereoselective assay has been utilized for quantification of total **1** in plasma samples in more than ten clinical studies [14].

A stability study was also performed to give an indication of the possible photodegradation of (±)-**1** during sample preparation. Two sets of 0.5 μg/ml samples were prepared with one set being exposed to light and the other covered

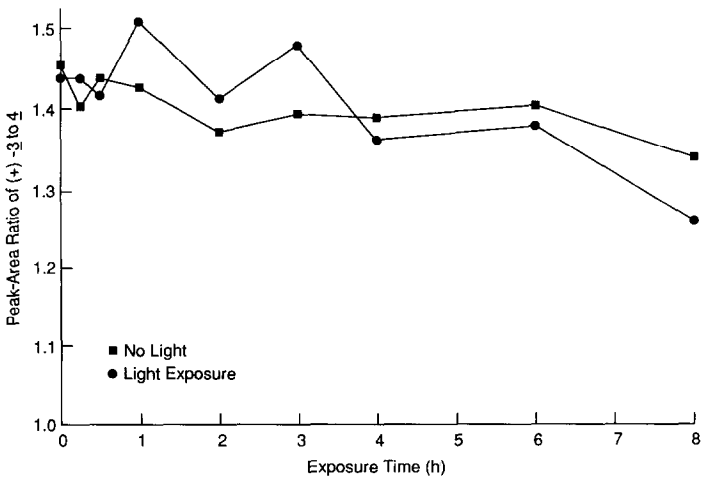

Fig. 2. Graphical results of photodegradation study for (+)-**1**.

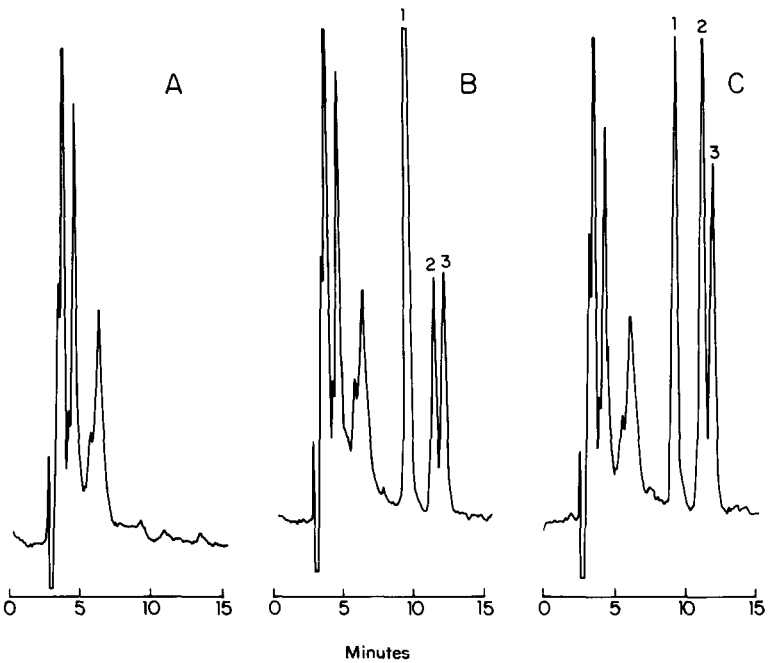

Fig. 3. Representative chromatograms of (A) blank plasma, (B) blank plasma (1 ml) spiked with 0.5 μg of (±)-**1** and 1.25 μg of I.S., and (C) subject plasma obtained 2 h after intravenous administration of (±)-**1** and spiked with 1.25 μg of I.S. Peaks: 1 = NEA derivative of internal standard; 2 = NEA derivative of (−)-**1**; 3 = NEA derivative of (+)-**1**.

with aluminum foil. At various time points (0, 0.25, 0.5, 1, 2, 3, 5, 6, and 8 h), a sample was removed from each set and placed in the refrigerator. All samples were analyzed using the stereoselective assay together. No significant degradation of (+)-**1**, as can be seen in Fig. 2, occurred for at least 6 h. A similar profile was seen for (−)-**1**.

Fig. 3. shows representative chromatograms from plasma samples using the extraction and derivatization methods describes above. Method validation showed no significant variation for inter-day ($n = 6$) or intra-day ($n = 8$) assays with correlation coefficients, calculated by linear regression analysis, for both standard curves exceeding 0.9995. The coefficient of variation (%) of the mean values for each calibration concentration was $< 10\%$. Quality control samples were assayed with each standard curve and (±)-**1** was found to be stable at least for five months when stored at −20°C.

Assay specificity was confirmed by testing the predose plasma which showed no interfering peaks. The precision of the method was demonstrated by the low intra-day and inter-day variability of standard curves and quality control samples (Table I). The accuracy of the method (Table II) based on inter-day quality control standards was 104% for both enantiomers at 0.25 μg/ml, and 99.2 and 97.6% for (±)-**1** and (−)-**1** enantiomers at 1.25 μg/ml.

TABLE I

VALIDATION DATA FOR THE DETERMINATION OF ENANTIOMERIC COMPOSITION OF (±)-**1** IN PLASMA

Concentration of racemic **1** (μg/ml)	Coefficient of variation (%)			
	Intra-day (n = 5)		Inter-day (n = 8)	
	(+)-**1**	(−)-**1**	(+)-**1**	(−)-**1**
0.05	4.3	4.2	6.8	3.2
0.10	3.1	4.0	3.6	4.3
0.25	3.0	2.9	9.4	6.4
0.50	2.3	2.3	6.0	4.5
1.25	3.5	2.3	4.0	4.8
2.50	4.8	3.5	6.4	6.4

TABLE II

INTER-DAY VARIABILITY FOR QUALITY CONTROL SAMPLES SPIKED WITH (±)-**1** (n = 5)

Nominal concentration (μg/ml)	Mean calculated concentration (μg/ml)		Coefficient of variation (%)	
	(+)-**1**	(−)-**1**	(+)-**1**	(−)-**1**
0.25	0.26	0.26	7.7	7.1
1.25	1.24	1.22	6.7	5.8

The stereoselective assay developed has been utilized to determine the enantiomeric composition of **1** in plasma samples of human subjects dosed with (±)-**1**. Results from an intravenous dose proportionality study are illustrated in Fig. 4 which shows the mean plasma concentration–time profiles of (+)- and (−)-**1** following oral administration of 25-, 100-, and 250-mg doses of (±)-**1**. In addition to human samples, this method is also applicable for many biological fluids of animal species (rat, dog, and monkey).

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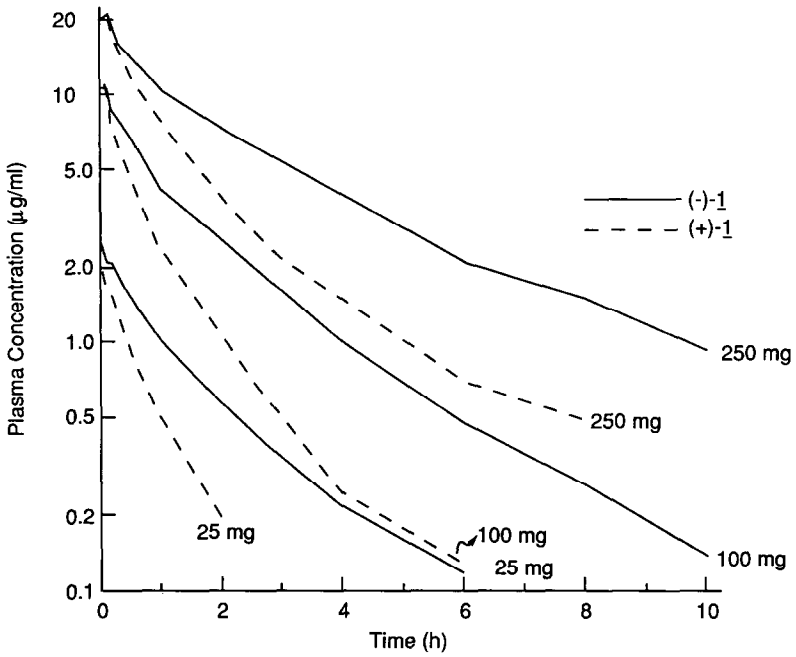

Fig. 4. Plasma concentration–time profiles for (−)-**1** and (+)-**1** after intravenous dosing of human subjects with (±)-**1**.

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