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Note

High-performance liquid chromatographic determination of 4-[(3-(4-acetyl-3-hydroxy-2-propylphenoxy)propyl)sulfonyl]-γ-oxobenzenebutanoic acid and metabolites in human plasma

C.M. ROTHWELL, J.Y.-K. HSIEH* and W.F. BAYNE

Merck Sharp & Dohme Research Laboratories, West Point, PA 19486 (U.S.A.)

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The leukotrienes constitute a group of peptidolipids derived from arachidonic acid through the action of the 5-lipoxygenase enzyme system and are believed to be mediators of allergic–inflammatory reactions, including human bronchial asthma [1]. The leukotrienes have been shown to produce bronchoconstriction at low concentrations in healthy individuals [2–4], as well as in individuals with asthma [5]. Recent experiments indicate that asthmatics are hyper-reactive to leukotrienes, specifically leukotriene D_4, compared to the reactivity of healthy, non-asthmatic individuals [6–8]. In addition, it has been shown that leukotrienes promote mucous production from human airways [9], while inhibiting mucociliary clearance [10]. Asthma attacks are characterized by both of these conditions.

4-[(3-(4-Acetyl-3-hydroxy-2-propylphenoxy)propyl)sulfonyl]-γ-oxobenzenebutanoic acid (I, Fig. 1) has been found to be a specific receptor antagonist of leukotriene D_4 [11] and may be useful in the treatment of human bronchial asthma, as well as in the treatment of cystic fibrosis, chronic bronchitis or allergic rhinitis. A new high-performance liquid chromatographic (HPLC) method for the determination of I and its metabolites, II and III (Fig. 1), in plasma is reported. The method has been developed to determine the pharmacokinetic profiles of these compounds in man. All three compounds can be reliably quantified with a detection limit of 50 ng/ml in plasma.

EXPERIMENTAL

All reagents were analytical grade. Chromatographic solvents were HPLC grade. All standards were supplied by Merck Frosst Research Labs. (Montreal, Can-

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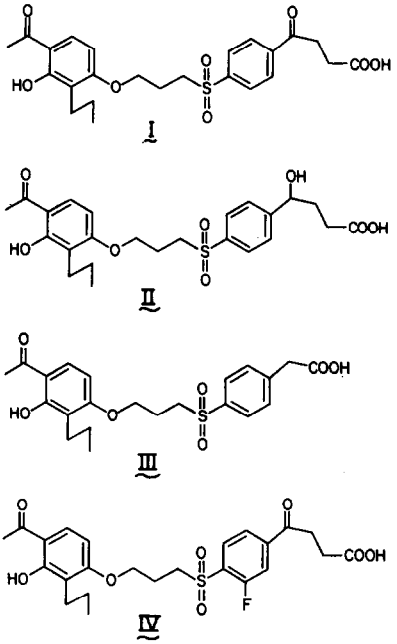

Fig. 1. Chemical structures of compounds I–IV.

ada). Stock standard solutions of I and II were 1.0 mg/ml and were prepared in a mixture of ethanol–water (1:1, v/v). The stock standard solution of III was 1.0 mg/ml and was prepared in ethanol. Standards, dilutions of these stock standard solutions, were prepared in ethanol. The stock standard solution of the internal standard (I.S.), IV, was 100 μg/ml and was dissolved in ethanol. Dilutions of this stock standard were also prepared in ethanol.

Apparatus

The isocratic HPLC separation was performed using a Perkin-Elmer Series 4 liquid chromatograph (Norwalk, CT, U.S.A.). Injections were made with a Perkin-Elmer ISS-100 autosampler. An Applied Science (State College, PA, U.S.A.) direct-connect 3 cm × 2.1 mm guard column packed with Whatman Co:Pell ODS (octadecylsilane groups bonded to 30–38 μm glass beads, Clifton, NJ, U.S.A.) was used, along with a 3-μm Supelcosil LC-18, 15 cm × 4.6 mm I.D. analytical column (Supelco, Bellefonte, PA, U.S.A.). Peaks were detected by monitoring absorbance at 280 nm (0.005 a.u.f.s.) with a Kratos Spectraflow 773 detector (Westwood, NJ, U.S.A.). Data were generated by a Spectra-Physics SP-4270 integrator (Santa Clara, CA, U.S.A.)

The pH of the mobile phase (0.85% orthophosphoric acid, pH 2.5–acetonitrile, 55:45, v/v) was adjusted to 3.1 with triethylamine, and the mobile phase was delivered at a flow-rate of 1.5 ml/min at ambient temperature. Total time for analysis was 12.0 min.

Clinical samples

Healthy male subjects received incremental 5-min intravenous infusions of 35.0, 35.0, 52.5 and 70.0 mg of I. Plasma was collected prior to drug treatment (predose) and at 0, 1, 2, 5, 10, 15, 30 and 60 min post the end of each infusion. Plasma samples were stored at −15°C until analysis.

Sample preparation and extraction

Plasma determined for I, II and III was prepared by placing 1.0 ml of plasma, 0.1 ml of ethanol, 0.1 ml of working I.S. (IV, 20 μg/ml), 0.5 ml of 1 *M* sodium acetate (pH 3.0) and 4 ml of methyl *tert.*-butyl ether in a polypropylene tube. Samples were vortex-mixed for 10 min and then centrifuged for 5 min at 2000 *g*. The methyl *tert.*-butyl ether was separated from the lower aqueous phase by freezing the aqeuous phase in a −60°C acetone bath and pouring off the methyl *tert.*-butyl ether. The methyl *tert.*-butyl ether was then dried in a vortex evaporator. The residue was reconstituted with 1.0 ml of mobile phase and vortexed for 10 s. The sample was then transferred to an autosampler vial and 50 μl were injected on the HPLC system.

Calibration standards were prepared by adding different amounts (0.1–10 μg) of I, II and III, 2 μg of I.S. (IV) and 0.5 ml of 1 *M* sodium acetate (pH 3.0) to 1 ml of human control plasma. Sample extraction and HPLC anaysis were carried out as described previously. Concentrations of I, II and III were calculated from the linear regression equation of the daily calibration curve constructed by plotting the peak-area ratios of I, II and III to the I.S. (IV).

RESULTS AND DISCUSSION

A reversed-phase HPLC method using UV detection at 280 nm has been developed for the determination of I and its metabolites II and III in plasma. Under the chromatographic conditions utilized, I, II and III were completely separated from one another as well as from the I.S., IV. Retention times for I, II, III and the I.S. (IV) were 8.3, 5.0, 7.6 and 10.2 min, respectively. Representative chromatograms of predose subject plasma and plasma collected after the administration of I are shown in Fig. 2. No interfering peaks at the retention times of I, II, III or IV were seen in the predose plasma.

Plasma was extracted using the method described above. Plasma was buffered at pH 3, 4, 5, 6 and 7 in order to determine the optimum pH for extraction efficiency. A pH of 3 or 4 produced better recoveries of I, II and III from plasma than a pH of 5, 6 or 7, with pH 3 giving the most accurate and narrowest range of recoveries for the three compounds. Various extracting solvents were tried, including methyl *tert.*-butyl ether, methylene chloride, 10% isopropanol in hexane, ethyl acetate, benzene and methyl ethyl ketone. Extraction with methyl *tert.*-butyl ether at pH 3 gave recoveries of 97–104% for I, II, III and I.S. (IV) from spiked plasma.

Removal of interfering plasma peaks was attempted in several ways. Plasma was extracted using Bond-Elut cartridges with ethylsilane (C_2), octadecylsilane (C_{18}) or silica packings. The liquid–solid extraction methods were not successful

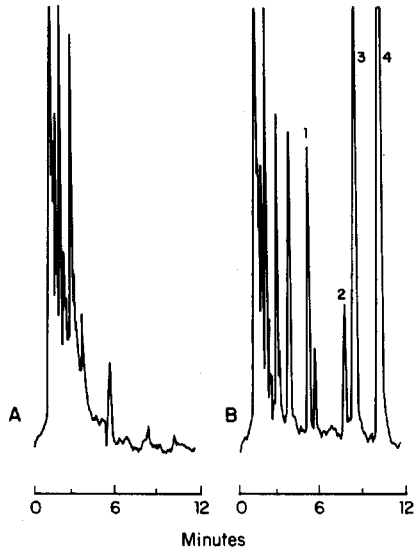

Fig. 2. Representative chromatograms of (A) predose subject plasma, (B) subject plasma collected 10 min after an infusion of 35.0 mg of I with 0.1 ml of 20 μg/ml I.S. (IV) added. Peaks: 1 = II; 2 = III; 3 = I; 4 = I.S. (IV).

because interfering peaks were collected with I. Various mobile phase compositions, addition of ion-pairing reagent and use of gradient-elution conditions were unsuccessful in separating I, II and III from interfering plasma peaks. Analytical columns (octadecylsilane, 3 μm) produced by various manufacturers and of various lengths were also tested. A 15 cm × 4.6 mm I.D. column from Supelco produced the best separation of I, II, III and IV from one another, as well as from interfering plasma peaks.

The method for determining I, II and III in plasma was evaluated for intra-day and inter-day variation. Standard curves ranged from 100 ng/ml to 10 μg/ml. Coefficients of variation were less than 10% (see Table I). The intra-day and inter-day correlations ($n = 5$) calculated using linear regression were 0.9999 for I, II and III. Quality-control samples containing I, II and III were prepared at concentrations representing the high (3.5 μg/ml) and low (0.3 μg/ml) ends of

TABLE I

INTRA-DAY AND INTER-DAY VARIATION ($n = 5$) for I, II and III

Concentration (μg/ml)	Relative standard deviation (%)					
	I		II		III	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
0.10	2.60	7.91	6.38	7.96	8.24	5.96
0.25	3.49	4.76	3.55	3.17	5.63	4.12
1.00	2.17	9.40	1.88	4.71	2.09	6.73
5.00	3.09	2.86	7.39	4.54	3.24	3.02

TABLE II

PLASMA CONCENTRATION OF I, II AND III FOLLOWING INTRAVENOUS INFUSION OF I AT CONCENTRATION SPECIFIED

N.D. = not detected under conditions specified in Experimental.

Time (min)	Concentration (μg/ml)											
	Infusion 1 (35.0 mg)			Infusion 2 (35.0 mg)			Infusion 3 (52.5 mg)			Infusion 4 (70.0 mg)		
	I	II	III	I	II	III	I	II	III	I	II	III
Predose	0	0	0	0	0	0	0	0	0	0	0	0
0	1.61	N.D.	N.D.	N.D.	N.D.	0.10	3.15	0.20	N.D.	3.69	0.25	0.18
1	2.56	0.10	N.D.	2.96	0.10	0.10	2.81	0.32	N.D.	4.99	0.40	0.22
2	2.80	0.16	N.D.	3.58	0.17	0.12	3.14	0.42	N.D.	5.08	0.59	0.25
5	2.14	0.34	N.D.	1.86	0.39	0.25	2.48	0.44	0.19	2.99	0.82	0.51
10	0.82	0.34	0.16	0.50	0.34	0.36	1.07	0.51	0.38	0.85	0.73	0.79
15	0.24	0.24	0.25	0.21	0.26	0.40	0.44	0.36	0.50	0.37	0.45	0.87
30	N.D.	N.D.	0.22	N.D.	0.11	0.30	N.D.	0.10	0.40	N.D.	0.16	0.66
60	N.D.	N.D.	N.D.	N.D.	N.D.	0.17	N.D.	N.D.	0.17	N.D.	N.D.	0.26

the standard curve. Intra-day and inter-day coefficients of variation ($n=5$) were less than 6% for the quality-control samples.

The method developed for the determination of I, II and III in plasma has been shown to be reproducible and reliable with a detection limit of 50 ng/ml of plasma for I, II and III. Table II shows levels of I, II and III in plasma from one subject after receiving incremental 5-min intravenous infusions of 35.0, 35.0, 52.5 and 70.0 mg of I. In general, the concentration of I increased until approximately 2 min post the end of the infusion at which time levels of I decreased. Low levels of II were seen at 0–1 min post the end of the infusion. The highest levels of II were seen at 5–10 min post the end of the infusion. Levels of III also followed the general pattern of increasing over time and then decreasing. Maximum levels of III were observed at 15 min post the end of the infusion.

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