GLC Determination of Ibuprofen [*dl*-2-(*p*-Isobutylphenyl)propionic Acid] Enantiomers in Biological Specimens

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Abstract □ To study the behavior of the *d*- and *l*-isomers of ibuprofen in humans, a method for the determination of the individual enantiomers in plasma and urine was required. A specific procedure was developed based on *(a)* benzene extraction of the acidified specimens, *(b)* TLC of the benzene extract residue, *(c)* formation of the *l*-α-methylbenzylamides of the materials eluted from the chromatograms, and *(d)* quantification of the resulting diastereoisomeric amides by GLC in conjunction with flame-ionization detection. When using a 1-ml aliquot of the specimen, the method is sensitive to 1 μg of each enantiomer/ml of plasma or urine. As compared to simple aqueous solutions, the average recoveries of the enantiomers from plasma and urine ranged from 94 to 96%. Mass spectrometric analyses, in conjunction with GLC, confirmed the specificity of the method for the intact enantiomers. The procedure was applied successfully to drug absorption studies in humans. After oral administration of the racemic mixture, the predominant enantiomer in peripheral circulation and excreted in urine was of the *d*-configuration.

Keyphrases □ Ibuprofen—GLC determination of enantiomers in biological specimens □ GLC—determination, ibuprofen enantiomers in biological specimens

Ibuprofen[1] [*dl*-2-(*p*-isobutylphenyl)propionic acid] (I) is reported (1, 2) to be one of the most potent orally active anti-inflammatory, antipyretic, and analgesic agents of a large number of substituted 2-phenylalkanoic acids based on a variety of tests in animals. The extensive toxicological and biochemical studies (3–5) in animals and humans (6) have been comprehensively reviewed (7).

Paper chromatographic (8) and GLC (5, 9) methods have been used to study the absorption, metabolism, and excretion of I in animals and humans. None of these procedures separated the individual enantiomers of the intact drug from each other. After oral administration of I to humans, the major metabolites excreted in urine were found to be dextrorotatory (1, 5). To study the behavior of the *d*- and *l*-isomers of I in humans (10), a specific GLC method for determining the individual enantiomers of the intact drug in plasma and urine was developed.

EXPERIMENTAL

Reagents and Materials—The I used in this study was synthesized[2] and the *d*- and *l*-isomers were resolved. Tridecanoic acid[3], *l*-α-methylbenzylamine[4], and hydrocarbon-stabilized chloroform[5] were used as supplied. The stock solution of 1,1′-carbonyldiimidazole[4] (II) (65 mg/ml) in hydrocarbon-stabilized chloroform was prepared fresh daily. Stock solutions of I, the *d*-isomer of I (III), the *l*-isomer of I (IV), and *n*-tridecanoic acid (V) in ethanol (100 μg/ml) were stored in glass containers. All other solvents were analytical reagent grade. Phenyl methyl silicone fluid (OV-17) on 60–80-mesh Gas Chrom Q[3] and chromatography plates coated with a 250-μm layer of silica gel F-254[6] were used as supplied.

Instrumentation—A two-speed reciprocating shaker[7] was used for shaking the samples in a horizontal position. A mixer[8] was used to aid in preparing the α-methylbenzylamide derivatives. GLC measurements were made with a gas chromatograph[9], equipped with a hydrogen flame-ionization detector and a −0.2–1.0-mv recorder[10]. All cylinders of gases used for chromatography (*i.e.*, helium, hydrogen, and oxygen) were fitted with filters containing molecular sieve 4A.

TLC Conditions—All TLC was conducted on silica gel F-254; the plates were developed (ascending) in 10% (v/v) acetic acid in toluene. The separated materials were visualized by irradiation of the plates with a short wavelength (254 nm) UV lamp or by spraying with 0.05% (w/v) bromocresol green in isopropanol[6]. Under these conditions, I, III, IV, and V have similar R_f values, *i.e.*, 0.40–0.42.

GLC Conditions—GLC was conducted on a U-shaped glass column (1.5 m × 3 mm i.d.) of 3% (w/w) OV-17 on 60–80-mesh Gas Chrom Q. All newly prepared columns were preconditioned at 250° for 1 hr without carrier gas flow and for 16 hr with a carrier gas flow of 10 ml/min. During analysis, the column, injection port, and detector block were maintained isothermally at 220, 245, and 260°, respectively. Helium, hydrogen, and oxygen flow rates were 90, 70, and 350 ml/min, respectively. Under these conditions, the *l*-α-methylbenzylamide derivatives of III, IV, and V have retention times of 8.0, 9.0, and 12.4 min, respectively (Figs. 1 and 2).

Synthesis of Standard Materials—Place 200 mg of III in a 200-ml round-bottom flask containing 25 ml of benzene. Slowly add 25 ml of thionyl chloride and reflux for 1 hr. Remove the solvent and excess reagent under reduced pressure (15 mm) at 40°. Dissolve the residue in 25 ml of benzene and remove under reduced pressure (15 mm) at 40°. Repeat the addition and removal of benzene. Dissolve the residue in 25 ml of chloroform and add 10 ml of chloroform containing 1 ml of *l*-α-methylbenzylamine. Stir for 30 min at 24°. Transfer the solution to a separator and extract successively with 10 ml of 1 *N* aqueous hydrochloric acid and two 10-ml volumes of water. Transfer the chloroform layer to a fresh round-bottom flask and remove the solvent under reduced pressure. Recrystallize the residue from aqueous methanol.

Synthesize standard materials for identification of the *l*-α-methylbenzylamide derivatives of IV and V utilizing the same general reaction conditions as described for III. Recrystallize the residues from hexane.

Assay Procedure—*Preparation of Standards*—Pipet aliquots of the I ethanol stock solution equivalent to 10, 20, 35, 50, and 65 μg into glass-stoppered centrifuge tubes. Add an aliquot of the V stock solution equivalent to 50 μg and evaporate to dryness with a gentle stream of nitrogen gas. Add 1 ml of control plasma or urine to each centrifuge tube and mix well with a mixer. Prepare an appropriate blank. Extract all standards in the same manner as described later for the plasma or urine samples.

Preparation of Samples—Pipet 0.5 ml of the V stock solution (100 μg/ml) into a series of 15-ml glass-stoppered centrifuge tubes and evaporate to dryness with a gentle stream of nitrogen. Place 1 ml of plasma or urine in each tube. Add 0.25 ml of 1 *N* aqueous hy-

[1] Motrin, The Upjohn Co.; and Brufen, Boots Co., Ltd.
[2] Boots Drug Co., Nottingham, England.
[3] Applied Science Labs, State College, Pa.
[4] Aldrich Chemical Co., Milwaukee, Wis.
[5] Matheson, Coleman and Bell, Milwaukee, Wis.
[6] Brinkmann Instruments, Westbury, N.Y.
[7] Eberbach & Sons, Ann Arbor, Mich.
[8] Scientific Industries, Queen's Village, N.Y.
[9] F & M model 400, Hewlett-Packard Co., Avondale, Pa.
[10] Honeywell Electronik 15.

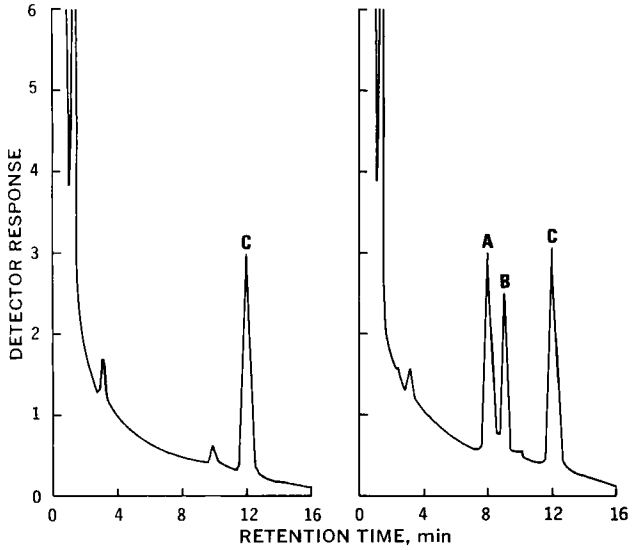


Figure 1—*Gas–liquid chromatograms of human plasma extracts. Left: normal plasma specimen. Right: plasma specimen from subject at 1 hr after single-dose oral administration of 800 mg of ibuprofen. Key: A,* d-*enantiomer; B,* l-*enantiomer; and C,* n-*tridecanoic acid internal standard.*

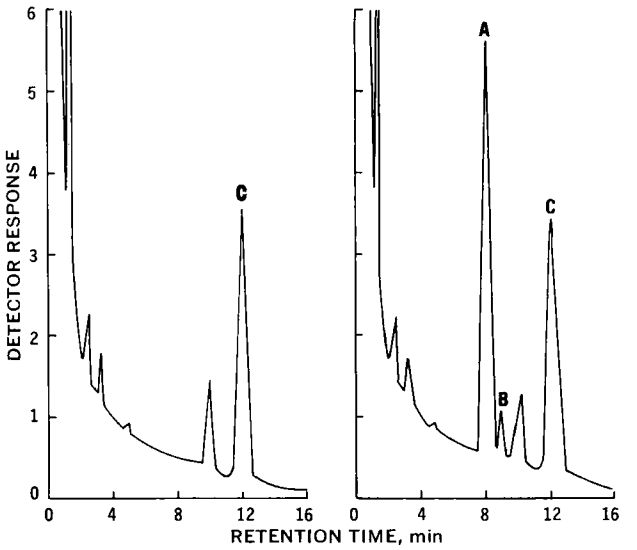


Figure 2—*Gas–liquid chromatograms of human urine extracts. Left: normal urine specimen. Right: urine specimen from subject during 0–4-hr time interval after single-dose oral administration of 800 mg of ibuprofen. Key: A,* d-*enantiomer; B,* l-*enantiomer; and C,* n-*tridecanoic acid internal standard.*

drochloric acid and 5 ml of benzene and shake in a horizontal position for 10 min. Centrifuge for 10 min at 2000 rpm. Transfer a 4-ml aliquot of the benzene layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with a gentle stream of nitrogen. Wash down the walls of the centrifuge tube with 0.5 ml of chloroform and evaporate to dryness with nitrogen.

Reconstitute the benzene extract residues from the standards and samples in 50 μl of chloroform and spot on the silica gel F-254 plates. Ascendingly develop each plate for a distance of 165 mm, air dry all chromatograms at room temperature (24°), and visualize the zones by irradiation with a 254-mm lamp. Scrape the zones corresponding to I and V into glass-stoppered centrifuge tubes. Add 1 ml of 1 *N* aqueous sodium hydroxide and mix thoroughly. Add 1 ml of 2 *N* aqueous hydrochloric acid and 5 ml of benzene and shake for 10 min in a horizontal position. Then centrifuge for 10 min at 2000 rpm. Transfer 3 ml of the benzene layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with nitrogen.

Add 0.1 ml of II reagent to each benzene extract residue. Rotate each tube to permit the reagent to contact the lower 2.5 cm of the centrifuge tube wall; allow the reagent to react for 5 min. Then add 10 μl of acetic acid and mix. Add 50 μl of *l*-α-methylbenzylamine, mix well, and allow the solution to react for 20 min. Inject a 1–5-μl aliquot for analysis into the chromatograph.

Calculations—The peak heights for the *l*-α-methylbenzylamide derivatives of III, IV, and V are measured. Peak height ratios are obtained by dividing the peak height of the III and IV amides by the peak height of the internal standard amide. Calibration curves from known concentrations of III and IV in plasma or urine are prepared by plotting peak height ratios *versus* free acid concentration, expressed as micrograms per milliliter of plasma or urine. Values for unknown concentrations of III and IV in plasma or urine specimens, obtained in the same manner, are then read directly from the graphs or calculated from the slopes of the standard curves.

Drug Administration to Humans—Informed written consent was obtained from each of three normal human male volunteers prior to participation in this study. All subjects were between 26 and 48 years; they ranged in body weight from 63.6 to 72.7 kg and in height from 1.62 to 1.81 m. All subjects were fasted for 16 hr prior to drug administration. Each then received an 800-mg dose of I as sugar-coated compressed tablets, and food was withheld for an additional 4 hr.

Blood specimens (10 ml) were withdrawn in heparinized syringes at 0, 1, 2, 4, and 8 hr after drug administration. The plasma was harvested and stored at −18°. Total urine specimens were collected for 12 hr prior to drug ingestion and at predetermined time intervals from 0 to 24 hr after drug administration. All specimens were stored at −18° until assay.

RESULTS AND DISCUSSION

Synthesis and Identification of *l*-α-Methylbenzylamide Derivatives of Ibuprofen Enantiomers and Internal Standard—Earlier investigations (9) showed that II was a useful reagent for the facile esterification of I in extracts of human plasma. The imidazolide intermediate of I formed very rapidly (≤1 min) and was highly reactive. A series of samples containing known amounts of III, IV, and V was prepared to determine the optimal reaction times for imidazolide formation and for amide formation from the imidazolides and *l*-α-methylbenzylamine. In studies with III and IV, known amounts of the *l*-α-methylbenzylamide derivative of V were added as the internal standard. Similarly, in studies with V, known amounts of the *l*-α-methylbenzylamide derivatives of III and IV were added as the internal standard.

The results indicated that imidazolide formation from III, IV, and V was complete within 1 min. Amide formation from the imidazolides of III and IV was complete within 20 min, while amide formation from the V intermediate was complete almost instanta-

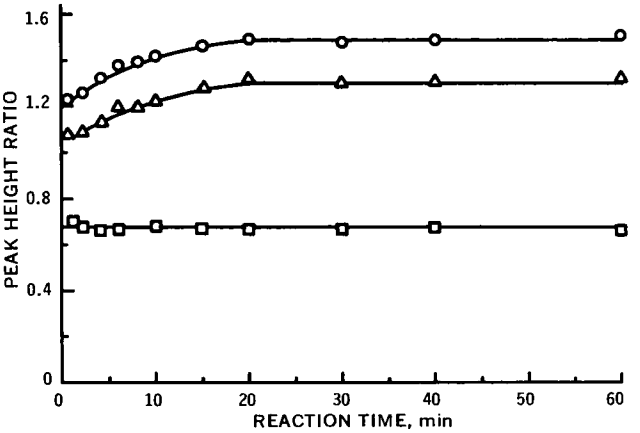
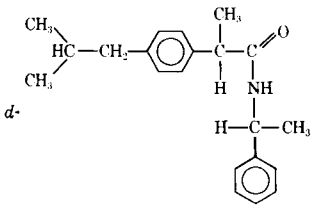
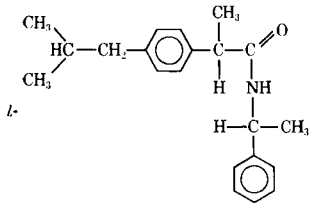
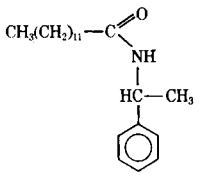


Figure 3—*Effect of reaction time* (l-α-*methylbenzylamine*) *on formation of amide derivatives of ibuprofen enantiomers and* n-*tridecanoic acid (reaction time of 1,1′-carbonyldiimidazole in chloroform = 5 min). Key:* ○, d-*enantiomer;* △, l-*enantiomer; and* □, n-*tridecanoic acid.*

Table I—Structural Formulas and Physical Properties of *l*-α-Methylbenzylamide Derivatives of Ibuprofen Enantiomers and *n*-Tridecanoic Acid

Structure[a]	Empirical Formula	Mol. Wt. Calc.	Mol. Wt. Found[b]	Analysis, % Calc.	Analysis, % Found	Melting Point	GLC Retention Time, min[c]
 *d*-	$C_{21}H_{27}NO$	309.43	309	C 81.51 H 8.79 N 4.52	81.45 8.70 4.39	109–110°	8.0
 *l*-	$C_{21}H_{27}NO$	309.43	309	C 81.51 H 8.79 N 4.52	81.48 9.03 4.46	96–97°	9.0
	$C_{21}H_{35}NO$	317.49	317	C 79.43 H 11.11 N 4.41	79.79 11.42 4.35	72–73°	12.4

[a] *d*- and *l*- refer to the optical rotation of the free acids used as starting materials. [b] Molecular weight of parent ion as determined by GLC in conjunction with mass spectrometry. [c] GLC conditions as described in *Experimental* section.

neously (Fig. 3). Reaction times of 5 min for imidazolide formation and of 20 min for amide formation were selected for convenience. Ancillary studies showed that at least 50 μl of *l*-α-methylbenzylamine was necessary to form the amides of III and IV quantitatively in the concentration range of 0–50 μg/ml of human plasma or urine (Fig. 4).

Synthesis of standard materials indicated that the *l*-α-methylbenzylamides of II, IV, and V were white crystals at room temperature (Table I). GLC, using a solid sample injector, indicated that the materials submitted for elemental analyses were greater than 99% pure. IR and mass spectrometric analyses, before and after GLC, supported the proposed structures and confirmed that the amides chromatographed as the intact molecules.

Identification of *N,N'*-Di-*l*-α-methylbenzylurea—During assay development, a major peak (R_T 10.1 min) unrelated to the individual enantiomers or internal standard was observed in the gas–liquid chromatograms from samples prepared with II and *l*-α-methylbenzylamine. Mass spectrometric analysis of the material(s) in the chromatographic peak suggested that the major component was the symmetrically disubstituted α-methylbenzyl derivative of urea. GLC and mass spectrometric analyses of *N,N'*-di-*l*-α-methylbenzylurea, synthesized directly from II and *l*-α-methylbenzylamine (11), showed that it was identical to the material found in the major peak. As described in the assay procedure, acetic acid (10 μl) was added to react with the excess II. Under these reaction conditions, little or no material responding in the assay as *N,N'*-di-*l*-α-methylbenzylurea was observed.

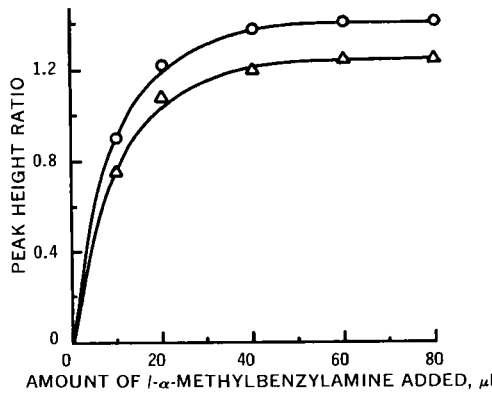


Figure 4—*Relationship between amount of* l-α-*methylbenzylamine added and formation of amide derivatives of ibuprofen enantiomers (reaction time of 1,1'-carbonyldiimidazole in chloroform = 5 min; reaction time of* l-α-*methylbenzylamine = 20 min). Key:* ○, d-*enantiomer; and* △, l-*enantiomer.*

Selection of Internal Standard—Pilot studies, using 2-(*p*-isobutylphenyl)acetic acid as an internal standard, showed that the *l*-α-methylbenzylamide derivative had a retention time of about 9.5 min as compared to 8.0 and 9.0 min for the *l*-α-methylbenzylamide of III and IV, respectively. As shown in Figs. 1 and 2, benzene extracts of plasma and urine specimens from normal human subjects contained interfering materials with approximately the same retention time (R_T 10.0 min) as the *l*-α-methylbenzylamide derivative of 2-(*p*-isobutylphenyl)acetic acid. Based upon *(a)* extraction efficiency into benzene, *(b)* mobility in the TLC solvent system, *(c)* formation of an *l*-α-methylbenzylamide derivative, and *(d)* GLC separation from III, IV, and endogenous materials present in extracts of plasma and urine, V was subsequently selected to replace the 2-(*p*-isobutylphenyl)acetic acid as the internal standard.

TLC of Plasma and Urine Extracts—Pilot studies were conducted with various TLC solvent systems to separate endogenous materials extracted from human plasma and urine that had relatively long GLC retention times (*i.e.*, 34 and 36 min, respectively). A solvent system composed of 10% (v/v) acetic acid in toluene separated the endogenous materials from I, III, IV, and V. The total time required for GLC of each plasma or urine sample was reduced from 40 to 16 min.

Assay Sensitivity and Specificity—At a sensitivity of 1.6×10^{-10} amp/mv, 0.87 μg of III and 0.95 μg of IV as the *l*-α-methylbenzylamides produced full-scale responses. However, under the assay conditions described, the lower limit of detection sensitivity for III and IV in extracts of human plasma or urine is 1.0 μg/ml of the original sample aliquot. This value is based on a sample signal equivalent to 2% of full-scale response. Under the assay conditions

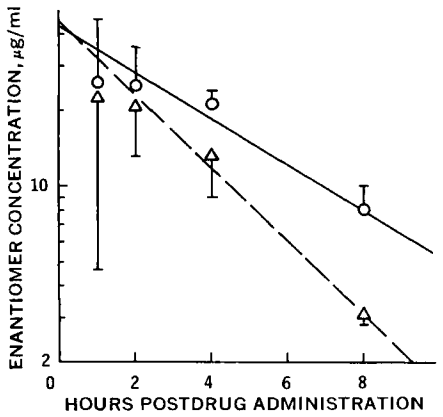


Figure 5—*Average* (±SD) *plasma concentrations of ibuprofen enantiomers* versus *time in humans* (n = *3*) *after single-dose oral administration of 800 mg of drug. Key:* ○, d-*enantiomer; and* △, l-*enantiomer. Bars indicate standard deviations.*

described, a linear relationship between detector response and concentration is obtained for III and IV over the range of 0–80 μg/ml. Quantification from a standard curve has been adequate. Analysis of plasma and urine specimens from drug-treated human subjects, using GLC in conjunction with mass spectrometry, has shown that the material responding to the assay is identical to known III- and IV-*l*-α-methylbenzylamide derivatives.

Recovery Experiments—Known amounts of III, IV, and V in ethanol were evaporated to dryness in centrifuge tubes; then water, plasma, or urine was added. The samples were thoroughly mixed and extracted with benzene. All extract residues were chromatographed on silica gel F-254, derivatized, and analyzed *via* GLC. The results indicated that, in the 5–35-μg/ml concentration range, recoveries of III and IV from plasma (94.7 ± 9.3 and 95.8 ± 11.1%, respectively) and urine (96.3 ± 2.7 and 93.8 ± 4.4%, respectively) were essentially quantitative as compared to simple aqueous samples.

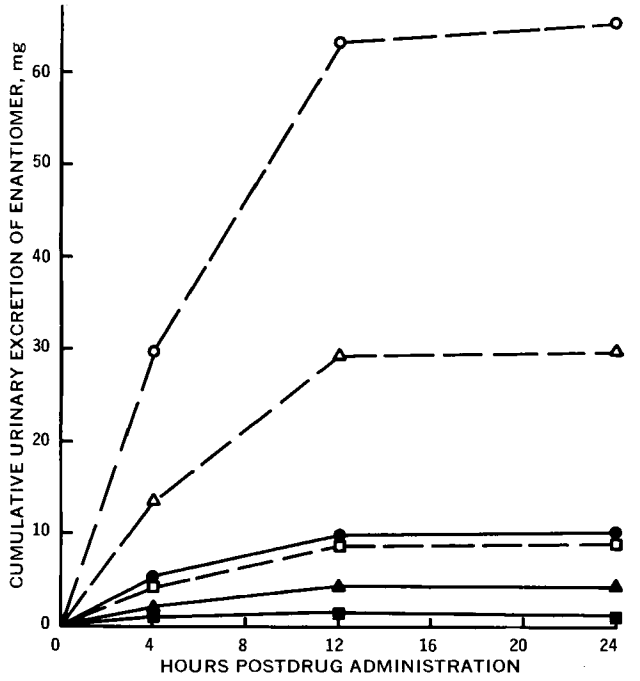


Figure 6—*Cumulative urinary excretion of ibuprofen enantiomers in humans after single-dose oral administration of 800 mg of drug (open symbols* = d-*enantiomer; closed symbols* = l-*enantiomer). Key:* ○,●, *Subject WW;* △,▲, *Subject PK; and* □,■, *Subject RT.*

Plasma and Urine Levels of Ibuprofen Enantiomers in Humans—Results from the measurement of plasma III and IV concentrations in three normal human subjects, after single-dose oral administration of 800 mg of I, demonstrated the utility of the analytical methodology (Fig. 5). Peak mean (±*SD*) levels of III (25.9 ± 19.6 μg/ml) and IV (22.6 ± 17.9 μg/ml) were observed at 1 hr after drug administration, indicating rapid drug absorption. As estimated graphically from the average plasma drug concentrations, the plasma drug disappearance half-lives for III and IV were 3.34 and 2.01 hr, respectively. The concentration ratio of III to IV increased progressively with time (from 1.17 at 1 hr to 2.65 at 8 hr), suggesting a stereospecific metabolism, excretion, or isomeric inversion.

Results from the measurement of III and IV in urine (Fig. 6) showed that III was the predominant isomer of the intact drug excreted. During the 0–24-hr time interval after drug administration, the concentration ratio of III to IV increased from 5.6 to greater than 9.0. The combined results from these investigations showed that the GLC method could be used for evaluating the pharmacokinetics and stereospecificity of metabolism, excretion, or isomeric inversion for the individual enantiomers of ibuprofen in humans.

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