The answer to the second question may be that the acute lethal toxicity (LD_{50}) is a very nonspecific type of biological response which depends upon nonspecific interactions with biopolymers or macromolecules. It is well known that compounds of higher degrees of symmetry usually have higher melting points and/or boiling points, indicating a greater degree of binding force with the neighboring molecules.

CONCLUSIONS

An apparent relationship was found between the degree of molecular symmetry and the acute oral lethal toxicity in animals as represented by the LD_{50}. This approach has some obvious limitations including the uncertainty in conformation assignments for large molecules and the lack of comparable data on large numbers of compounds of interest of higher order symmetry groups. Furthermore, the approach may not be directly applicable to drugs or substances that exert very specific biological activity by interaction with enzymes, such as antimetabolites, or to drugs that are rapidly metabolized in the body to other species with altered biological activity.

In general, however, this approach may have uncovered a new parameter which should be considered in the design of potentially specific, nontoxic drugs. In a given chemical classification, for example, this parameter may be included along with other physical-chemical parameters such as lipophilicity and steric and electronic factors utilized in current approaches to structure-activity correlations. Group theory can also be taken one step further by using quantum mechanical calculations of molecular interaction in the case of a specific process where a significant amount of information is known about the interaction between the drug and the receptor. Additional studies on specific pharmacological groups or agents will further demonstrate the validity and usefulness of this working hypothesis.

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GLC Determination of Plasma Levels of Enantiomers of α-[4-(1-Oxo-2-isoindolinyl)phenyl]propionic Acid

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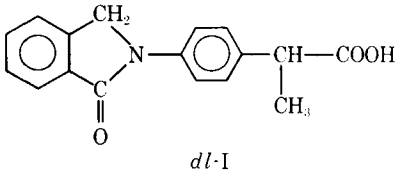
Abstract □ A quantitative GLC determination of each α-[4-(1-oxo-2-isoindolinyl)phenyl]propionic acid enantiomer in human plasma after oral administration of the racemate is described. After extraction and purification of the extract through partition steps, the substances were converted to the diastereoisomeric amides *via* the acid chlorides. These derivatives were separated and quantitated by GLC. The sensitivity limit is 0.3 μg of each enantiomer/ml plasma. In the concentration range of 0.62–5.00 μg/ml plasma, the percent recovery (± standard deviation) of the *d*- and *l*-enantiomer was 67.51 ± 3.11 and 67.44 ± 3.14, respectively, whereas the coefficients of variation of the ratios between these recoveries and those of the internal standard were 1.03 and 1.05%, respectively.

Keyphrases □ α-[4-(1-Oxo-2-isoindolinyl)phenyl]propionic acid—GLC determination of plasma levels of enantiomers □ α-Phenylpropionic acid derivatives—GLC determination of plasma levels of enantiomers □ GLC—determination, enantiomers of α-[4-(1-oxo-2-isoindolinyl)phenyl]propionic acid, plasma levels, humans

dl-α-[4-(1-Oxo-2-isoindolinyl)phenyl]propionic acid (*dl*-I) is a promising analgesic and anti-inflammatory agent. The chemistry (1) of this substance and its biological activities (2) were reported. Compound *dl*-I was approximately 20 times as active as phenylbutazone in inhibiting carrageenin-induced edema of the rat paw, 15 times as active as phenylbutazone in the granuloma pouch test, and about 20 times as potent as phenylbutazone in preventing adjuvant-induced arthritis in the rat. When using the parameter of phenylquinone-induced writhing in the mouse, the compound proved equipotent with indomethacin, 95 times more potent than phenylbutazone, and 50 times more potent than aspirin.

Pharmacokinetics studies (3) showed that the substance in its racemic modification was completely and rapidly absorbed from the GI tract when administered in capsules to healthy volunteers (peak plas-



dl-I

ma levels between 30 min and 2 hr) and rapidly eliminated ($t_{1/2\beta}$ between 2.16 and 5.40 hr). These values were obtained from plasma level determinations by techniques (radioactivity and GLC) that do not distinguish between the enantiomers and, therefore, reflect the parameters for the *dl*-form.

Since the high degree of stereospecificity of the anti-inflammatory action of several pairs of α-methylarylacetic acid enantiomers is known (4), the determination of the pharmacokinetic parameters of each enantiomer of *dl*-I after administration of these substances and of their racemate was desirable. The results should indicate whether the two enantiomers are characterized by different pharmacokinetic parameters and whether any interaction exists regarding their absorption, elimination, and metabolism. Therefore, a method for the determination of the plasma levels of the two enantiomers was required. The method described here is based on the conversion of the two enantiomeric acids, extracted from plasma, into their chlorides and reaction of the latter with *l*-α-methylbenzylamine; the resulting two diastereoisomeric amides can be easily separated and determined by GLC.

EXPERIMENTAL

Reagents—*l*-α-Methylbenzylamine[1] and other solvents[2] or reagents[3] were used as purchased.

Reference Compounds—*dl*-I and *dl*-II were synthesized by the methods described in the literature (1). *d*-I, *l*-I, and *d*-II were obtained from their racemates by repeated fractional crystallizations of their salts with *l*-α-methylbenzylamine; the optical purity of these compounds, ascertained by the GLC and NMR spectra of their amides with *l*-α-methylbenzylamine, was greater than 98%. These amides and the amide of *dl*-II with cyclohexylamine were prepared by the following method. The acid (0.2 g) was suspended in anhydrous benzene (25 ml) and treated with thionyl chloride (25 ml), the suspension was refluxed until complete dissolution (about 1 hr), and the solvent and thionyl chloride were then evaporated. The evaporation was repeated twice after addition of anhydrous benzene (25 ml). To the residue, dissolved in anhydrous chloroform (25 ml), the amine (1 ml) in chloroform (10 ml) was added in small portions. After 30 min of agitation at room temperature, the solution was washed with 0.1 *N* HCl (10 ml) and then twice with water (10 ml). The organic phase was evaporated under vacuum and the residue was crystallized twice from the solvent indicated for each amide in Table I. Table I also reports the structural and empirical formulas and the physical characteristics of the reference compounds used in this work and gives the numbers by which these compounds are identified.

GLC—For the GLC determinations, a gas chromatograph[4] equipped with a flame-ionization detector (hydrogen = 50 ml/min, air = 300 ml/min) was utilized. A coiled glass column (length 2 m, i.d. 3 mm) packed with 1% OV-7[5] on acid-washed, silanized Chromosorb W (60–80 mesh) was used; the support was coated by the evaporation technique. Nitrogen was used as the carrier gas (flow rate = 90 ml/min). The temperatures were as follows: column, 280°; and injector port and detector, 300°. The retention times of the compounds are reported in Table I. Figure 1 shows a gas chromatogram of the reference compounds employed. In quantitative GLC determinations, the peak area obtained by multiplying the peak height by the width of the peak at half its height was used.

GLC Reference Compound Calibration Curves—The following reference compound solutions were prepared.

A. Quantitation standard solution: 50 μg *dl*-V/ml chloroform.

B. Stock solutions of reference compounds *d*-III and *l*-III: dissolve 25 mg *d*-III and *l*-III in 25 ml Solution A.

C. Stock solution of reference compound *d*-IV: dissolve 25 mg *d*-IV in 25 ml Solution A.

D. Working solutions: pipet 0.25, 0.5, 1.0, 1.5, and 2.0 ml of B and 1 ml of C in five 10-ml calibrated flasks and dilute to volume with A. Each solution contained 50 μg/ml of *dl*-V and 100 μg/ml of *d*-IV together with 25.0, 50.0, 100.0, 150.0, and 200.0 μg/ml of *d*-III and *l*-III.

GLC data for each solution of D gave the same linear relationship, passing through the origin, as was obtained by plotting the ratios of peak area *d*-III/peak area *dl*-V and peak area *l*-III/peak area *dl*-V as a function of the concentration of *d*-III and *l*-III, respectively. For each reference compound, a "specific peak area ratio" can, therefore, be calculated. For *d*-III, this is given by:

$$\text{specific peak area ratio} = \frac{\text{peak area } d\text{-III}}{\text{concentration } d\text{-III}} \times \frac{\text{concentration } dl\text{-V}}{\text{peak area } dl\text{-V}} \qquad \text{(Eq. 1)}$$

The same expression is used to calculate the specific peak area ratio for *l*-III and *d*-IV.

Quantitative Conversion of d-*I,* l-*I, and* d-*II into* d-*III,* l-*III, and* d-*IV*—The following standard solutions were prepared.

E. Stock solution of *dl*-I: dissolve 25 mg *dl*-I in anhydrous methanol (25 ml). The stock solution was refrigerated; fresh solutions were made monthly.

F. Stock solution of *d*-II (internal standard): dissolve 25 mg *d*-II in anhydrous methanol (25 ml). The stock solution was refrigerated; fresh solutions were made monthly.

G. Working solutions: pipet 0.25-, 0.5-, 1.0-, and 2.0-ml aliquots of E and 1-ml aliquots of F into four 10-ml calibrated flasks and dilute to volume with anhydrous methanol.

Conversion Procedure—Aliquots (0.1 ml) of each solution of G were transferred into four test tubes and evaporated at 40° under a stream of nitrogen; each sample thus contained 10.0 μg of *d*-II together with 1.25, 2.50, 5.0, and 10.0 μg of *d*-I and *l*-I, respectively. The residue was redissolved in anhydrous ethyl acetate (0.2 ml), treated with thionyl chloride (25 μl), and heated for 1 hr at 60° in stoppered test tubes. The solution was then evaporated at room temperature under a stream of nitrogen; anhydrous ethyl

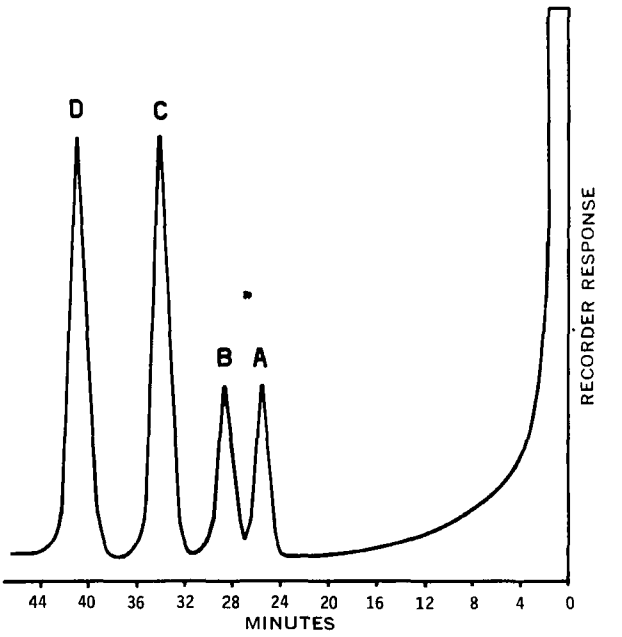
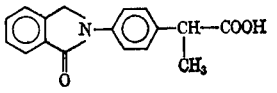
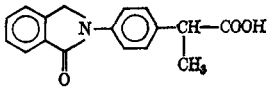
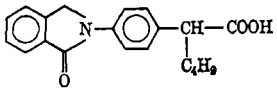
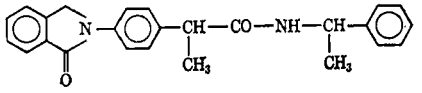
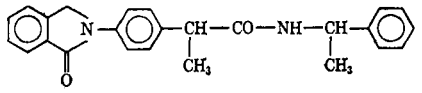
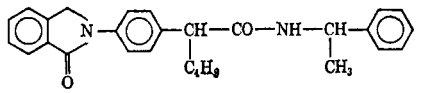
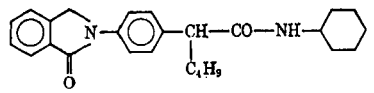


Figure 1—*Gas chromatogram of the reference compounds. Key: A,* d-*III; B,* l-*III; C,* dl-*V (quantitation standard); and D,* d-*IV (internal standard). Conditions were: 1% OV-7 on Chromosorb W (60–80); nitrogen flow rate, 90 ml/min; column temperature, 280°; and recorder chart speed, 30 cm/hr.*

[1] Aldrich Chemical Co.
[2] RS per pesticidi, Carlo Erba.
[3] RP, Carlo Erba.
[4] Carlo Erba, model GI.
[5] Applied Sciences Laboratories.

Table I—Physical Characteristics of Isoindolinylphenylpropionic Acids and Their Derivatives

Compound[a]	Structural Formula	Molecular Formula	Crystallization Solvent	Melting Point	Retention[b] Time, min
d-I		$C_{17}H_{15}NO_3$	Ethanol	209–212°	—
l-I		$C_{17}H_{15}NO_3$	Ethanol	209–212°	—
d-II		$C_{20}H_{21}NO_3$	Ethanol	163–164°	—
d-III		$C_{25}H_{24}N_2O_2$	Benzene	197–198°	24.9
l-III		$C_{25}H_{24}N_2O_2$	Methanol	235–236°	28.2
d-IV		$C_{25}H_{30}N_2O_2$	Ethanol–water	131–133°	40.2
dl-V		$C_{26}H_{32}N_2O_2$	Ethanol–water	188–189°	33.6

[a] The *d*- and *l*-symbols of amides refer to the rotatory power of the starting acids; the optically active amides were prepared from *l*-α-methylbenzylamine.
[b] For GLC conditions, see *Experimental*.

Table II—Conversion Yields of *d*-I, *l*-I, and *d*-II

Micrograms of Compound in Each Sample			Average Conversion[a] ± *SD*, %			Average Conversion Ratios[a] ± *SD*		
d-I	*l*-I	*d*-II	*d*-I	*l*-I	*d*-II	*d*-I/*d*-II	*l*-I/*d*-II	*d*-I/*l*-I
1.25	1.25	10.0	89.20 ± 1.04	87.40 ± 0.76	91.92 ± 0.71	0.970 ± 0.0045	0.950 ± 0.0071	1.020 ± 0.0039
2.50	2.50	10.0	89.65 ± 3.00	88.02 ± 3.31	92.05 ± 4.19	0.974 ± 0.0074	0.957 ± 0.0080	1.018 ± 0.0046
5.0	5.0	10.0	94.70 ± 1.51	93.02 ± 1.39	97.35 ± 0.97	0.973 ± 0.0091	0.955 ± 0.0100	1.018 ± 0.0045
10.0	10.0	10.0	91.10 ± 2.72	89.27 ± 2.73	93.10 ± 2.01	0.978 ± 0.0083	0.959 ± 0.0099	1.020 ± 0.0040
Overall average ± *SD*						0.974 ± 0.0074	0.955 ± 0.0080	1.019 ± 0.0040

[a] Mean of four determinations.

acetate (0.1 ml) was added and the evaporation was repeated. The residue was redissolved in anhydrous ethyl acetate (0.1 ml), and *l*-α-methylbenzylamine (10 μl) was added. After 15 min at room temperature, the solvent and amine were vacuum evaporated at 60° under nitrogen. Ethyl acetate (0.1 ml) was added and the evaporation was repeated. Then 0.1 ml of Solution A was added and a suitable amount of the solution (1–5 μl) was injected into the chromatograph. By using each peak, an experimental specific peak area ratio was calculated by Eq. 1; the ratio between this value (×100) and the theoretical one obtained as described in the preceding paragraph gives the conversion percentage of *d*-I, *l*-I, and *d*-II into *d*-III, *l*-III, and *d*-IV, respectively. The results obtained by this procedure (mean of four determinations for each sample) are reported in Table II; they permit the conclusion that, since the average conversion ratios *d*-I/*d*-II and *l*-I/*d*-II fall in a narrow range (0.974 ± 0.007 and 0.955 ± 0.008, respectively), *d*-II is a suitable internal standard to control the conversion of *d*-I and *l*-I. The same data also show that the mean conversion yield of *d*-I is higher than that of *l*-I, the ratio between them being 1.019 ± 0.004. TLC examination [on silica gel[6] in the solvent system of benzene–ethyl acetate–acetic acid (130:24:30)] of the products obtained by the conversion procedure shows that the conversion is quantitative and that no side-products are formed; losses may occur only during the evaporation steps.

Recoveries of *d*-I, *l*-I, and *d*-II from Human Plasma—*Partition Characteristics*—The determination of partition coefficients between aqueous buffers with different pH and organic solvents showed that *d*-I and *l*-I are quantitatively extracted with 4 volumes ether from aqueous solution at pH < 2 and with 0.1 volume 0.1 *N* NaOH from ether. In stability studies, these compounds were stable in 0.1 *N* HCl and 0.1 *N* NaOH; the same partition and stability characteristics were shown by *d*-II used as the internal standard to check the recovery of *d*-I and *l*-I.

Extraction Procedure—Each Solution G (0.1 ml) was taken to dryness at 40° under a stream of nitrogen and treated with 2 ml

[6] Stratocrom SIF RS, Carlo Erba.

Table III—Recoveries of *d*-I, *l*-I, and *d*-II from Plasma

Micrograms of Compound Added to Each Milliliter Plasma Sample			Average Recovery[a] ± *SD*, %			Average Recovery Ratios[a] ± *SD*		
d-I	*l*-I	*d*-II	*d*-I	*l*-I	*d*-II	*d*-I/*d*-II	*l*-I/*d*-II	*d*-I/*l*-I
0.62	0.62	5.0	69.35 ± 2.46	69.10 ± 2.48	72.12 ± 2.88	0.962 ± 0.0066	0.958 ± 0.0048	1.003 ± 0.0032
1.25	1.25	5.0	66.40 ± 2.01	66.35 ± 1.91	70.52 ± 2.38	0.942 ± 0.0099	0.941 ± 0.0080	1.001 ± 0.0065
2.50	2.50	5.0	67.75 ± 3.32	67.90 ± 3.43	71.25 ± 2.87	0.951 ± 0.012	0.953 ± 0.014	0.998 ± 0.0039
5.0	5.0	5.0	66.53 ± 4.06	66.40 ± 4.20	69.13 ± 3.59	0.962 ± 0.010	0.956 ± 0.013	1.002 ± 0.0032
Overall average ± *SD*						0.954 ± 0.0098	0.953 ± 0.010	1.001 ± 0.0046

[a] Mean of six determinations.

human plasma; each sample thus contained 5 μg of *d*-II and 0.62, 1.25, 2.5, and 5.0 μg of *d*-I and *l*-I/ml plasma, respectively. After equilibration at 37°, plasma samples were acidified by addition of 4 ml 0.1 *N* HCl and extracted for 15 min with ether (40 ml) by mechanical shaking. The organic phase was washed with 5 ml distilled water and reextracted with 0.1 *N* NaOH (5 ml). From the aqueous phase after washing with ether (40 ml) and acidification with 1 *N* HCl, *d*-I, *l*-I, and *d*-II were extracted by mechanical shaking for 15 min with 40 ml ether. After washing with distilled water (5 ml), the ether was taken to dryness at 30° under a stream of nitrogen. The residue obtained was then partitioned between methanol (1 ml) (containing 2 drops water) and hexane (3 ml) by shaking for 15 min. The hexane phase (upper) was removed by aspiration and discarded, the methanol phase was taken to dryness, and derivatization of the residue was accomplished as described under *Conversion Procedure*. The final derivatives were chromatographed under the experimental conditions described previously. The percent recoveries of *d*-I, *l*-I, and *d*-II from plasma were calculated by the ratios (×100) between the experimental specific peak area ratios obtained for each sample by this procedure and the theoretical value of the same parameter deduced from the calibration curves. The recoveries (mean of six determinations) of increasing amounts of *d*-I and *l*-I and a constant amount of *d*-II from plasma are reported in Table III. On the basis of the results obtained in recovery determinations, the following method was developed to determine *d*-I and *l*-I present in human plasma.

Determination of *d*-I and *l*-I in Unknown Samples of Human Plasma—Compound *d*-II (internal standard) (10 μg) was added to 2 ml human plasma; the sample was acidified with 4 ml 0.1 *N* HCl and submitted to the extraction and conversion procedures described. Plasma samples were stored frozen at −20° until assayed; during 3 weeks of storage under these conditions, no change was noted in the concentrations of *d*-I and *l*-I.

RESULTS AND DISCUSSION

Derivative Preparation—The derivative most suitable for GLC separation of *d*-I from *l*-I was found by the preparation of the amides of these compounds with *l*-α-methylbenzylamine. Esters of the same enantiomers with optically active alcohols (*e.g.*, 1,2-methyl-1-butanol), even if easier to prepare, were not separable by GLC. OV-7 (1%) proved the most efficient stationary phase to achieve the separation of the diastereoisomeric amides mentioned; OV-17 can produce separation of these compounds but with less satisfactory results.

Method Precision—The data of Table III show that the amounts of *d*-I and *l*-I recovered from plasma and calculated with reference to the recovery of *d*-II are significantly related (r = 0.999 for $p < 0.01$) to the amount added by the following linear relationship:

$$\text{amount recovered} = \text{amount added} \times b + a \qquad \text{(Eq. 2)}$$

For *d*-I, $b \pm SD = 0.964 \pm 0.0039$ and $a \pm SD = -0.037 \pm 0.022$ (a is not significantly different from zero for $p = 0.05$). For *l*-I, $b \pm SD = 0.962 \pm 0.0044$ and $a \pm SD = -0.031 \pm 0.025$ (a is not significantly different from zero for $p = 0.05$). These data and those of Table III indicate the improvement in precision with the internal standard technique in comparison to the procedure that

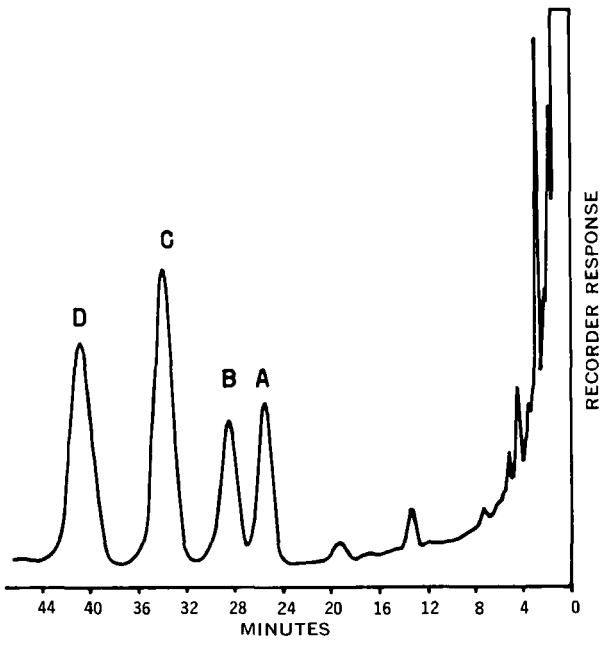


Figure 2—*Gas chromatogram of an extract of 2 ml human plasma of a subject treated with 200 mg of* dl-*I orally. Key and conditions: same as in Fig. 1.*

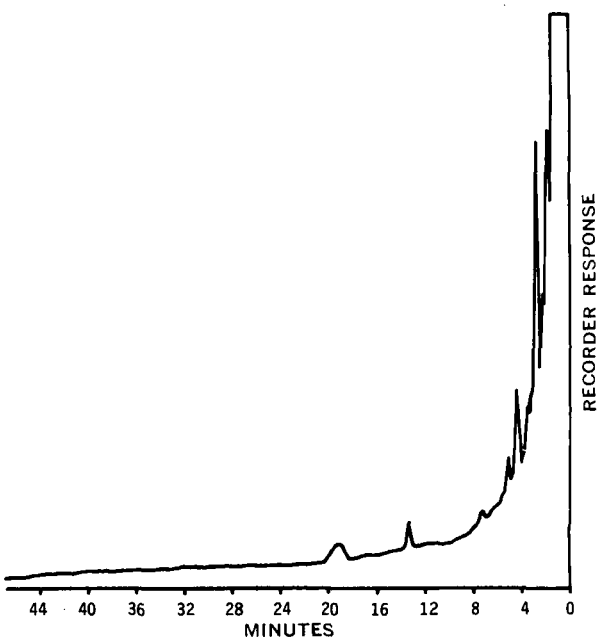


Figure 3—*Gas chromatogram of an extract of 2 ml blank human plasma. Conditions: same as in Fig. 1.*

Table IV—Plasma Levels (Micrograms per Milliliter) of *d*-I and *l*-I in a Subject Treated with 200 mg of *dl*-I Orally[a]

Hours	*d*-I	*l*-I	*A*	*B*	*A* − *B*
0.5	0.92	1.01	1.93	—	—
1.0	6.70	6.16	12.86	12.90	−0.04
1.5	6.94	5.46	12.40	12.60	−0.20
2.0	6.29	4.31	10.60	10.46	+0.14
3.0	4.84	3.03	7.87	8.03	−0.16
6.0	3.39	1.61	5.00	5.03	−0.03
8.0	2.55	1.10	3.65	3.68	−0.03
10.0	1.62	0.66	2.28	2.22	+0.06

[a] *A* = (*d*-I) + (*l*-I) plasma levels obtained by the present method. *B* = (*d*-I) + (*l*-I) plasma levels obtained by the method described in Ref. 3.

uses as reference recovery the mean recovery of each substance to be determined. The addition of the internal standard (*d*-II) to the initial plasma samples makes unnecessary the total recovery of extracts during the extraction procedure. Since (as already stated) the amounts of *d*-I and *l*-I in unknown plasma samples are deduced by reference to the recovery of the internal standard, the quantitation standard (*dl*-V) added at the end of the derivatization should not be necessary. However, it is used to check the efficiency of the extraction and conversion procedures.

Method Specificity—Figure 2 shows the gas chromatogram for the determination of *d*-I and *l*-I in the plasma extract of a subject treated with 200 mg of *dl*-I. Figure 3 represents a blank plasma extract of the same subject; in this chromatogram there are no peaks interfering with those of the substances under examination or with those of internal and quantitation standards. Such interferences are absent even if the equivalent of 4/100th of an extract of 4 ml plasma is injected.

Method Sensitivity—By using 4 ml of plasma in the extraction and derivatization and injecting 4/100th of the final solution, up to 0.3 μg of *d*-I and *l*-I/ml plasma can be determined.

Figure 4 shows the plasma levels of *d*-I and *l*-I in a subject treated with 200 mg of *dl*-I orally; these curves show that *d*-I and *l*-I are characterized by comparable absorption rates but different elimination half-lives[7]. Table IV reports the comparison between the sum (*d*-I) + (*l*-I) plasma levels deduced from the data of Fig. 4 and those obtained experimentally by using the same plasma samples in the extraction and derivatization to obtain this sum directly. This procedure (3) is based on the same extraction steps described in this paper but applies a conversion reaction (formation of 2,2,2-trifluoroethyl esters of *dl*-I) which does not permit a distinction between *d*-I and *l*-I plasma levels. Table IV shows the good accordance between the results obtained by these two methods.

[7] An extensive study on this difference will be published elsewhere.

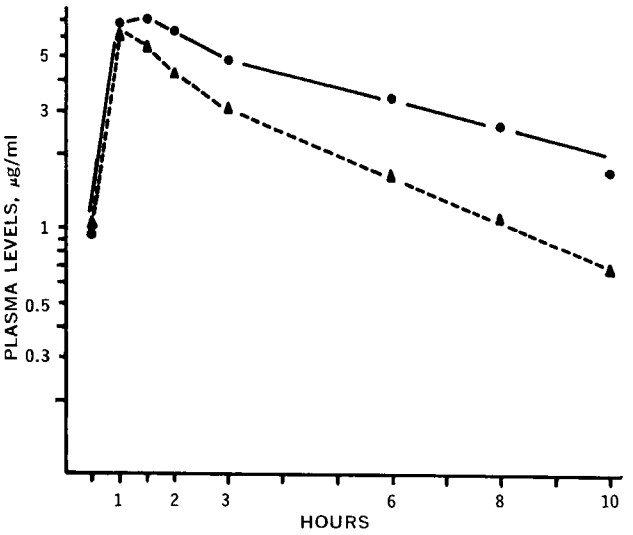


Figure 4—*Plasma levels (micrograms per milliliter) of* d-*I and* l-*I in a subject following administration of 200 mg of* dl-*I orally. Key:* ●—●, d-*I; and* ▲- - -▲, l-*I.*

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