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Optical isomerization of *R*(−)-clidanac to the biologically active *S*(+)-isomer in guinea-pigs

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Optical isomerization of clidanac (*RS*-6-chloro-5-cyclohexyl-1-indancarboxylic acid, I), an anti-inflammatory drug having a chiral centre in its molecule, was evaluated in guinea-pigs. After oral administration of *R*(−)-I, the biologically active *S*(+)-isomer was detectable in the plasma, in the early stages. At 3 h after dosing *R*(−)-I, the plasma contained above 90% of the *S*(+)-isomer. Little conversion of *S*(+)-I to *R*(−)-I was observed. This may account for the equivalent in vivo activities of *R*(−)- and *S*(+)-I in this species. Determination of the enantiomeric composition required derivatization of the enantiomers to their diastereomeric amides after which thin layer chromatography (t.l.c.) was used for the separation. The quantitative determination of the compounds so-separated was accomplished by in situ measurements of the u.v.-reflectance. The t.l.c.–u.v.-densitometric procedure was also used to determine the plasma concentration of I.

Clidanac (*RS*-6-chloro-5-cyclohexyl-1-indan- carboxylic acid, I) is a non-steroidal anti-inflammatory drug. In vitro inhibitory activity on prostaglandin synthesis resides entirely in the *S*(+)-enantiomer [*S*(+)-I] (Tamura et al 1981), which has more potent pharmacological activities than the *R*(−)-enantiomer [*R*(−)-I] in rats and mice (Kuzuna et al 1974). In guinea-pigs, however, the two enantiomers have almost equivalent in vivo activities (Kuzuna et al 1974), suggesting an in vivo conversion of *R*(−)-I to *S*(+)-I in this species, since the optical inversion of anti-inflammatory chiral α-arylpropionic acids to the (+)-enantiomers in animals and man has been reported (Kaiser et al 1976; Lan et al 1976; Bopp et al 1979; Tanaka & Hayashi 1980).

To demonstrate the in vivo conversion of the optical configuration of I, a thin layer chromatographic (t.l.c.) method was developed to determine the individual enantiomers as their diastereomeric amides. As indan compounds including I have absorption bands in the u.v. region, an in situ reflectometric quantitation of the amide derivatives separated by t.l.c. could be made. The data obtained using this method showed that the optical isomerization of *R*(−)-I to the *S*(+)-isomer does occur in guinea-pigs.

MATERIALS AND METHODS

Materials

RS-I was resolved via the quinine salt, as previously reported (Noguchi et al 1974). *R*(+)-α-Methylbenzylamine (99 + %) [*R*(+)-II] was purchased from Aldrich Chemical (Milwaukee), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (specially prepared reagent for peptide synthesis) from Nakarai Chemicals (Kyoto) and t.l.c. glass plates (20 × 20 cm) coated with a 0·25 mm layer of silica gel 60 F_{254} (Art. 5715) from E. Merck (Darmstadt). All other reagents and solvents were of reagent grade.

Syntheses of standard materials

S(+)-Clidanac *R*(+)-α-methylbenzylamide (*S,R*-III) was prepared as follows: *S*(+)-I (100 mg) was dissolved in CH_2Cl_2 (5 ml) and to this was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (82 mg) (Sheehan et al 1965) at 0 °C with stirring. After reacting for 30 min at 0 °C, *R*(+)-II (55 μl) was added and stirred for 60 min at

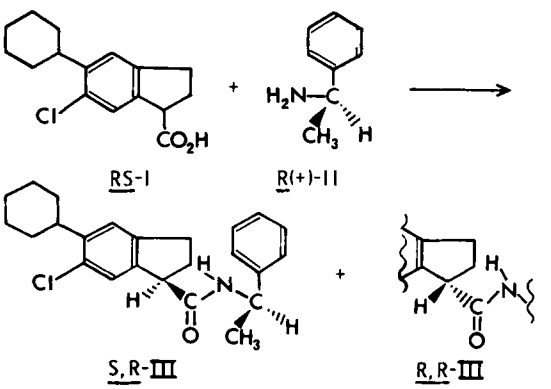

FIG. 1. Scheme of derivatization reaction of clidanac (I) with *R*(+)-α-methylbenzylamine [*R*(+)-II] to the diastereomeric amides (*S,R*- and *R,R*-III).

* Correspondence.

room temperature (20 °C). The solution was washed successively with water, 1 M HCl, 4% $NaHCO_3$, and water, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue was recrystallized from ethyl acetate to give colourless crystals (44 mg), m.p. 190–191 °C, $[\alpha]_D^{25.5°} = +19.3°$ (c = 0·15, $CHCl_3$). Anal. Calcd. for $C_{24}H_{28}NOCl$: C, 75·47; H, 7·39; N, 3·67. Found: C, 75·22; H, 7·41; N, 3·87. I.r. ν_{max} (Nujol) cm^{-1}: 1640 (CONH). U.v. λ_{max} (ethanol) nm: 284. N.m.r. (in $CDCl_3$) δ: 3·79 (1H, t, >CH-CONH), 5·14 [1H, m, CONH-CH(CH_3)-C_6H_5], 7·28 (5H, s, C_6H_5). M.s. *m/z:* 383, 381 (M^+), 235, 233 [M^+-CONH-CH(CH_3)-C_6H_5], 105 (C_6H_5-CH-CH_3).

The *R*(+)-α-methylbenzylamide of *R*(−)-I (*R,R*-III) was synthesized by utilizing the same reaction conditions as described for *S*(+)-I. The product was crystallized from ether-light petroleum to give 80 mg as colourless crystals, m.p. 87–90 °C and 145–148 °C (double melting point), $[\alpha]_D^{25.5°} = -2.8°$ (c = 0·324, $CHCl_3$). Anal. Found: C, 75·35; H, 7·65; N, 3·84. I.r. ν_{max} (Nujol) cm^{-1}: 1640. U.v. λ_{max} (ethanol) nm: 284. N.m.r. (in $CDCl_3$) δ: 3·80 (1H, t, >CH-CONH), 5·15 [1H, m, CONH-CH(CH_3)-C_6H_5], 7·23 (5H, s, C_6H_5). M.s. *m/z*: 383, 381, 235, 233, 105.

To produce a large quantity of these derivatives, *RS*-I was reacted with *R*(+)-II via the acid-chloride intermediate and the resultant amides were resolved by recrystallization.

Isolation and determination of I from blood plasma

Groups of 4–6 male STD-Hartley guinea-pigs (470–720 g) were, various times after oral administration of 20 mg kg^{-1} of *RS*-I or the enantiomers respectively, anaesthetized with ether and blood samples were withdrawn from the abdominal vein into heparinized syringes. Plasma samples (1 ml) were acidified by adding 0·25 ml of 1 M HCl, then extracted twice with 5 ml benzene and centrifuged, and the organic phase was evaporated to dryness. The residue was dissolved in exact volume of methanol, and the 10 μl aliquot was spotted in a circle, less than 5 mm in diameter, on a t.l.c. plate, and developed for approximately 16 cm with hexane–dioxane–acetic acid (20:6:1, v/v/v). The spots corresponding to I were measured with a Shimadzu dual wavelength-u.v.-densitometer CS-910 by a reflectometric procedure, under the following conditions: light-absorbing wavelength (λ_S), 280 nm; reference wavelength (λ_R), 360 nm; beam span, 1·25 × 1·25 mm; sensitivity, 1–10 mV (Shimadzu U-225 MCS recorder); scanning mode, zig-zag.

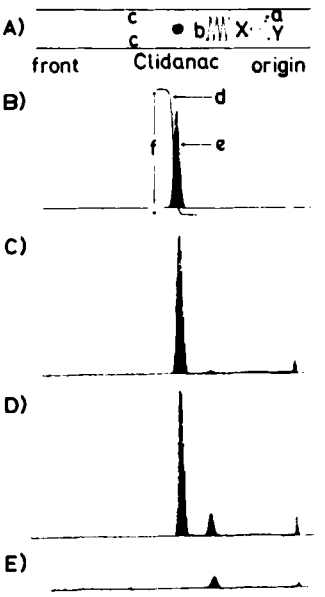

FIG. 2. Thin layer chromatograms of I. T.l.c. of benzene extract of the plasma was carried out with hexane–dioxane–acetic acid (20:6:1, v/v/v). Zone containing I separated on t.l.c. was measured by reflectometry at the dual wavelength of spot beam (a); an absorption maximum of I at 280 nm and no absorption at 360 nm. The zone was scanned with a along X axis while the stage carrying the t.l.c. plate was reciprocated (Y), the beam trace resulting in zig-zag (b). The reflectance, after background compensation (at point c), was recorded every time the beam passed the zone on the recorder as a spike. Distribution profile (e) of a zone area was thus obtained, and the integration curve (d) was obtained as a summation of each spike. The content in the area was calculated from the integration height (f). For particulars, see the paper of Yamamoto et al (1976). (A), visible spot of authentic I; (B), its u.v.-reflectance profile; (C), sample plasma; (D), plasma spiked with I; (E), control plasma.

Photomultiplier signals were finally led to an integrator, and by the integration heights, peak areas obtained from the reflectance measurements were determined (see Fig. 2).

Chiral derivatization and quantitation

To the residues of benzene extract from plasma (1 ml), 0·1 ml of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride 3 mg ml^{-1} CH_2Cl_2 solution was added and the preparation well mixed. The reaction mixture was allowed to stand for 30 min at 0 °C, and then 0·1 ml of *R*(+)-II 1 μl ml^{-1} in $CHCl_2$ was added and mixed, and allowed to stand for a further 60 min at room temperature. Then 3 ml of CH_2Cl_2 was added to the reaction mixture, and the preparation washed successively with 3 ml each of water, 1 M HCl, and water (aqueous phases were aspirated). The organic phase was evaporated to

dryness under N_2 stream, and the residue was dissolved in methanol. The aliquot (10 μl) was applied on a t.l.c. plate in the same manner as in the case of the free acid, and developed with benzene-acetonitrile (100:13, v/v). The spots separated were visualized under an u.v.-lamp and identified by cochromatographying authentic samples. The zone areas of *S,R*- and *R,R*-III were measured by zig-zag scanning reflectometry with the same wavelength (λ_S, 280 and λ_R, 360 nm) and operations as above.

RESULTS

T.l.c.-densitometry of I, and S,R-III and R,R-III

The chromatograms obtained with control plasma, plasma to which I had been added, and sample plasma are shown in Fig. 2. Endogenous materials interfering with the analysis of I were never detected in numerous control plasma samples.

The diastereomeric amides could be clearly separated from each other by t.l.c. with various solvent systems examined. Of a number of the solvent systems, benzene–acetonitrile (100:13) gave the best separation and R_F values; the method displayed baseline separation of *S,R*- and *R,R*-III (Fig. 3). In the reflectance spectrum of III, an absorption maximum occurred at 280 nm as well as in that of I. Fig. 3 also shows that the chromatograms were free from interfering peaks.

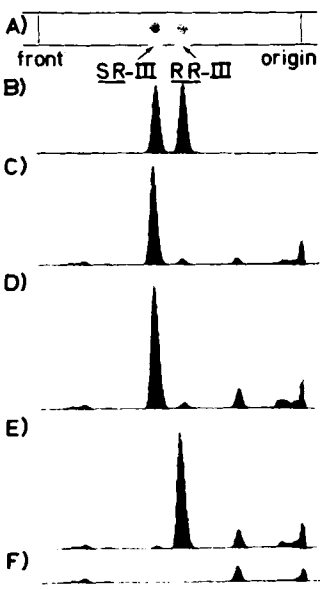

FIG. 3. Thin layer chromatographic separation of *S,R*-III and *R,R*-III, and their u.v.-reflectance profiles. Following derivatization of I extracted from plasma to *S,R*- and *R,R*-III, t.l.c. was carried out with benzene–acetonitrile (100:13, v/v). The determination of the compounds was accomplished by the same procedure and condition as in Fig. 2. (A), Authentic samples; (B), their u.v.-reflectance profiles; (C), sample plasma after dosing *R*(−)-I; (D), plasma spiked with *S*(+)-I; (E), plasma spiked with *R*(−)-I; (F), control plasma.

Calibration curves

Calibration curves were prepared by plotting the integration height versus various amounts of authentic samples by the identical procedure. The relationship between integration height and amount applied on the t.l.c. plate (after development) was linear over the range of 0·25–60 μg of I (r = 0·9990, slope = 34·6). Likewise, linear relationships were obtained for ranges 0·25–40 μg of *S,R*-III and *R,R*-III (r = 0·9999, slope = 29·2 and r = 0·9998, slope = 28·3, respectively). The straight lines calculated by the least square method passed through an origin, respectively. Also, day-to-day reproducibilities of their calibration curves were observed. The sensitivity limit of their detections was the respective 0·25 μg.

Recovery of I from plasma

For 2, 5, 10, 20, 50 and 80 μg of I spiked in 1 ml of plasma, the recoveries after extraction and densitometric measurement were 81·2 ± 8·0% (coefficient variation 9·8%), 77·5 ± 5·8 (7·5), 80·0 ± 3·9 (4·9), 92·7 ± 11·6 (12·5), 85·9 ± 1·6 (1·8) 91·4 ± 10·6 (11·6), respectively (mean with s.d. for 6 determinations).

Diastereomeric derivatization of S(+)- and R(−)-I

The recoveries of *S,R*- and *R,R*-III during extraction and derivatization process from plasma (1 ml) spiked with *S*(+)-, *R*(−)- or *RS*-I were determined. The maximum percent formation of III that could be obtained by using the carbodiimide as a condensation agent under the reaction condition described in *Methods* was 57% (Table 1). Variation in reaction time, temperature, solvent and the carbodiimide concentration did not increase the formation of III. However, the amine concentration affected the amide yield; the yield decreased with decreasing I concentration (Table 1). The formation of III was slightly favourable to *R*(−)-I, but as shown in Table 2, the ratio of *S,R*- and *R,R*-III produced was all but identical to the ratio of the enantiomers present in the original sample. The data presented in Tables 1 & 2 further show that the products could be prepared with little or no racemization; the resulting amides included a few percent of the respective diastereomer.

Table 1. Formation of the amide derivatives of I enantiomers. *RS*-I or the enantiomers was added to plasma (1 ml) with indicated concentrations. After benzene extraction of the plasma, I enantiomers were derived to the *R*(+)-II-amides by using the carbodiimide as a condensation reagent. Yields of their amide measured by t.l.c.-u.v.-densitometric method were calculated on a mole basis relative to I present in the original sample (n = 4).

Enantiomer added	μg	Composition obtained by derivatization (%) *S,R*-III	*R,R*-III	Yield (%) Mean (with s.d.)
S(+)	5	97·8	2·2	34·7 (8·7)
	20	92·7	7·3	42·7 (5·0)
	80	93·5	6·5	56·7 (4·9)
R(−)	5	0	100	36·6 (6·2)
	20	5·0	95·0	45·5 (9·1)
	80	3·3	96·7	57·0 (6·1)
RS	5	43·0	57·0	43·3 (0·9)
	20	47·8	52·2	48·8 (5·3)
	80	47·7	52·3	54·9 (11·1)

Plasma concentrations of I and the enantiomeric composition

The initial study, 24 h after oral administration of 1, 3 or 20 mg kg^{-1} of *RS*-I to each of 6 guinea-pigs, showed a dose-dependent increase in plasma concentrations; that is, 4·5 ± 0·3, 12·1 ± 0·6, and 74·2 ± 0·7 μg ml^{-1} (mean ± s.e.) respectively.

After dosing of 20 mg kg^{-1} of *S*(+)-, *R*(−)-, or *RS*-I, the absorption was rapid and the plasma concentrations increased with time. Those for *S*(+)- and *RS*-I were higher than that for *R*(−)-I (Fig. 4 *left*). The metabolites were not present in any of the plasma samples.

The distribution of the *S*(+)-composition in these plasma samples is shown in Fig. 4 *right*. In the plasma after dosing *R*(−)-I, the *S*(+)-form was found as early as 15 min. At this time, the *S*(+)/*R*(−) ratio had already reached 29·1/70·9. Thereafter, the ratio continuously increased to above 90% *S*(+)-I measured 3 h after dosing. On the contrary, after administration of *S*(+)-I, there was little conversion to *R*(−)-I. These observations substantiated by the *S*(+)- composition after dosing *RS*-I reflected the average behaviour of the two enantiomers.

Table 2. Ratio of *S,R*- and *R,R*-III formed by the carbodiimide method. A series of standard samples containing I enantiomers were prepared with indicated composition, and reactions with *R*(+)-II were then carried out in presence of the carbodiimide. Resultant diastereomer-composition was compared with the composition of the enantiomers present in the original sample.

Enantiomer composition* (%) *S*(+)-I	*R*(−)-I	Diastereomer composition (%) *S,R*-III	*R,R*-III
100	0	94·0	6·0
90	10	83·3	16·7
80	20	74·8	25·2
70	30	65·7	34·3
60	40	56·9	43·1
50	50	48·7	51·3
RS		49·4	50·6
40	60	39·1	60·9
30	70	31·2	68·8
20	80	23·0	77·0
10	90	14·8	85·2
0	100	6·7	93·3

* Total amount 20 μg.

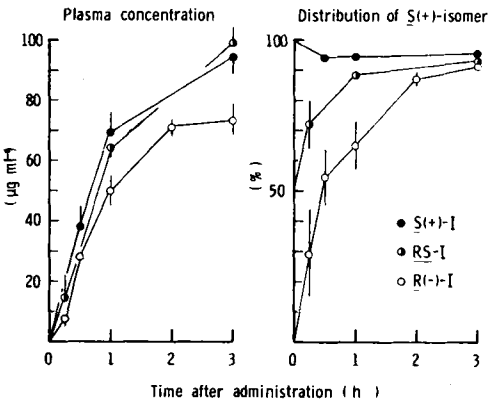

FIG. 4. Plasma concentrations of I (*left*) and percent distribution of the *S*(+)-isomer in the plasma (*right*). The plasma concentrations of I after oral administration of 20 mg kg^{-1} of *RS*-I or the individual enantiomers (see key at right side of figure) were determined following benzene extraction of acidified plasma. Each point represents mean ± s.e. for 4–5 determinations (*left*). Enantiomer composition of I in benzene extract was determined following derivatization to the diastereomeric amides. Data are presented as a percent distribution of the *S*(+)-composition in extracted I. Each point represents mean with s.d. (*right*).

DISCUSSION

The t.l.c.-u.v.-densitometric method allowed for a simple and accurate means of determining the concentration of I in plasma, and this availability made feasible studies on the pharmacokinetic behaviour of *S*(+)- and *R*(−)-I. This method was sufficiently reliable to determine μg-quantities of I, or *S,R*- and *R,R*-III. The plasma concentrations, 24 h after administration of *RS*-I, as measured by this method, were the same as those measured by radioassay (Tanayama 1973).

After administration of 20 mg kg^{-1} of *S*(+)- or *R*(−)-I, there were differences in their plasma level-time profiles (Fig. 4). This may be due to difference of the absorption rates from the gastrointestinal tract or to a faster elimination of *R*(−)-I than *S*(+)-I from plasma. Similarly, Kuzuna et al (1974)

have reported that R(−)-I showed preferential biotransformation in rats. Although stereochemistry no doubt affects the metabolism of drugs (Jenner & Testa 1973), it is not known whether such stereoselectivity in the metabolism of RS-I exists in the guinea-pig, since orally administered RS-I to guinea-pigs undergoes little biotransformation and maintains long-lasting plasma concentrations (Tanayama 1973).

To determine the ratio of S(+)- to R(−)-I in plasma, a chiral derivatization method was required. The I enantiomers obtained with the aid of water-soluble carbodiimide to their R(+)-II amides could then be separated by t.l.c. This method showed nearly equal reactivity of the two enantiomers. However, the amide yield did not exceed 60%, probably due to by-product formation, assumably acylurea(s) (Sheehan et al 1965), rather than to incompleteness of the reaction. Since unreacted I was not found in the chromatograms, and since a longer reaction time and higher amine concentration did not improve the yield, the by-product(s) seem to have been removed by washing with aqueous solutions, together with excess reagents. We attempted to derive I extracted from plasma quantitatively to the amide by such coupling methods as the acid chloride (Lan et al 1976), the imidazolide (Kaiser et al 1976), the active ester (Bodanszky & du Vigneaud 1959), or the mixed anhydride (Vaughan & Osato 1952). The reactions were not completed in a short time and sometimes a significant racemization of I occurred, or undesirable products interfering with the assay were produced. The low yield in the carbodiimide method was, however, compensated for by the high detection sensitivity of the u.v.-reflectance measurement in determining III derived from μg-order concentrations of plasma I.

After oral administration of 20 mg kg^{-1} of R(−)-I to guinea-pigs, the S(+)-isomer appeared in the plasma in the early stages. Above 90% of R(−)-I was converted to the S(+)-isomer as early as 3 h after dosing, suggesting that R(−)-I may have a chemical structure susceptible to an optical isomerization reaction or that some enzyme system catalysing such a reaction may be distributed with a high activity in the guinea-pig, or both. On the other hand, the S(+)-to-R(−) conversion, if any, appears to be much smaller than the R(−)-to-S(+) conversion, both in rate and extent. These findings indicate that the conversion of R(−)-I to the biologically active S(+)-form may contribute to the in vivo effect of R(−)-I.

During the process of the optical isomerization to S(+)-I, α-hydrogen of R(−)-I is considered to be eliminated as a proton, a proton and an electron, or a hydride ion. If the reaction proceeds by the first type reaction, the enzyme participating in this reaction may not require an organic co-factor; the carbanion (at C-1) must be formed. If the reaction proceeds by second or third mechanism, some organic co-factors such as flavin or NAD^+ may be necessary. In preliminary in vitro studies using various tissue preparations of guinea-pigs, however, R(−)-I was not converted to S(+)-I under some incubation conditions, even in presence of organic co-factors such as flavin or NAD^+, or metal ions, suggesting that such activation of the carboxyl group of R(−)-I as Wechter et al (1974) speculated may need to initiate the reaction. Recently, evidence for optical isomerization mechanism of ibuprofen was reported (Nakamura et al 1980). The authors using rat liver preparations and deuteriated compounds found that R(−)-ibuprofen was first activated to its CoA ester, then was racemized. Although the chemical structure of RS-I is not similar to ibuprofen, an α-methylpropionic acid analogue, it is possible that the same enzyme system or reaction mechanism may also be involved in the isomerization reaction of R(−)-I. Tanaka & Hayashi (1980) showed that stereospecific inversion of 2-(2-isopropylindan-5-yl)propionic acid in rats proceeds through the enolic form intermediate of the carboxylic acid. Thus, in the case of R(−)-I, the intramolecular transfer of α-hydrogen to carbonyl of activated R(−)-I may be first elicited by some enzyme system, then the racemization reaction proceeds through a carbanion intermediate.

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