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# Investigations on the *in vitro* racemization of thalidomide by high-performance liquid chromatography☆

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ABSTRACT

The racemization of thalidomide enantiomers was examined in various aqueous media. The determination of the enantiomeric ratio was carried out by high-performance liquid chromatography (HPLC) on a poly[(*S*)-N-(1-cyclohexylethyl)methacrylamide] stationary phase. A significant increase in the rate of thalidomide racemization was observed in human citric plasma when compared with incubations in buffer. Further investigations using physiological concentrations of human serum albumin (HSA) in phosphate buffer (0.067 *M*, pH 7.4) demonstrated the influence of albumin on racemization. This observation was confirmed by inhibition experiments with capric acid anions.

## INTRODUCTION

The former sedative thalidomide, 1,3-dioxo-2-[2′,6′-dioxopiperidin-3′-yl]-isoindol (Fig. 1a), which has been used in human therapy as the racemate, was withdrawn from the market at the beginning of the 1960s. The substance exhibits a selective toxicity in the embryo while being relatively non-toxic for the mother. The cause of these malformations is still unknown.

Earlier investigations on the teratogenicity of thalidomide demonstrated that after i.p. application in rats and mice only *S*-(−)-thalidomide was teratogenic [1]. However, it is known that thalidomide is sensitive to hydrolytic decomposition and that it undergoes racemization [2,3]. It has been previously reported that the half-life of racemization in phosphate buffer (0.067 *M*, pH 7.4) at 37°C is 2.7 h [3]. Previous studies in our laboratory showed that thalidomide reisolated from plasma 2 h after i.v. application of *S*-(−)-thalidomide is completely racemized (Fig. 1b) [4]. These results prompted the investigation of

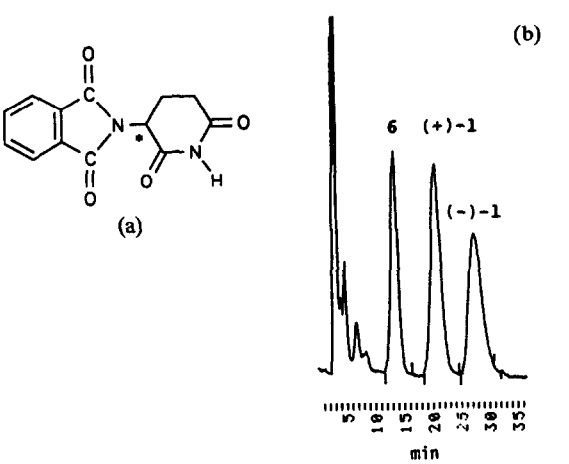


Fig. 1. (a) Structure of thalidomide. (b) High-performance liquid chromatogram of thalidomide extracted from rabbit plasma 2 h after i.v. application of *S*-(−)-thalidomide (1 = thalidomide; **6** = internal standard) Chromatographic conditions: poly[(*S*)-N-(1-cyclohexylethyl)methacrylamide] stationary phase; *n*-hexane–dioxane 70:30 (v/v); 1.0 ml/min; 230 nm (UV) [4] Abscissa time in min; ordinate, UV absorbance at 230 nm.

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the thalidomide racemization in further detail. In this paper we present racemization studies of thalidomide after incubation of the enantiomers in different aqueous media. The enantiomeric ratio after extraction from these media was determined by chiral HPLC.

## EXPERIMENTAL

### *Chemicals*

Racemic thalidomide was a gift from Grünenthal (Stolberg, Germany). Thalidomide enantiomers were separated on a preparative scale by low-pressure liquid chromatography on a poly[(*S*)-N-(1-cyclohexylethyl)methacrylamide] stationary phase [1] using tetrahydrofuran-*tert.*-butyl methyl ether (3:7, v/v) as the mobile phase. The purity of the enantiomers was determined by chiral HPLC and exceeded 99.5%.

Human serum albumin (HSA) (fraction V, essentially fatty acid free) and capric acid sodium salt were purchased from Sigma (Deisenhofen, Germany). The molecular weight of HSA was assumed to be 69 000 [5].

Human citric plasma was obtained from the Red Cross (Münster, Germany). Heparinized human plasma was prepared from blood samples of a healthy male volunteer by adding 5000 I.U. of heparin (Liquemin N 5000, Hoffmann–La Roche, Grenzach-Wyhlen, Germany) to 20 ml of blood. Rabbit plasma samples were kindly provided by Professor Dr. H. Ockenfels (University of Ulm, Ulm, Germany).

Tetrahydrofuran was purchased from Baker (Gross-Gerau, Germany) and redistilled over sodium. *tert.*-Butyl methyl ether, synthetic grade, was from Merck (Darmstadt, Germany). Dioxane, ethyl acetate and *n*-hexane were either from Baker or LiChrosolv reagents purchased from Merck. All other chemicals were obtained of the highest quality commercially available and used without further purification.

Buffer solutions were prepared in double-distilled, deionized water.

### *Apparatus*

The HPLC system consisted of a Knauer HPLC Pump 64 (Knauer, Bad Homburg, Germany), a Rheodyne sample injector (Model 7125, Rheodyne) equipped with a 20-$\mu$l loop, a variable-wavelength UV detector 655A (Merck–Hitachi, Darmstadt, Germany) and a D-2500 chromato-integrator (Merck–Hitachi).

### *Chromatographic conditions*

Thalidomide enantiomers were separated on a 250 mm × 4 mm I.D. poly[(*S*)-N-(1-cyclohexylethyl)methacrylamide] stationary phase which was immobilized on modified silica [6], equipped with a 30 mm × 4 mm I.D. LiChrospher 60 (10 $\mu$m particle size, Merck) guard column. The mobile phase was *n*-hexane–dioxane (7:3, v/v), the flow-rate 1.0 ml/min and the detection wavelength 230 nm.

### *Sample preparation*

Standard solutions of the thalidomide enantiomers were prepared in dioxane. Capric acid sodium salt was dissolved in ethanol (1.04 mg/ml). HSA solutions (fraction V, essentially fatty acid free) in phosphate buffer (0.067 *M*, pH 7.4) with a final (physiological) concentration of 4 g/100 ml were freshly prepared prior to the experiments.

### *Incubation conditions*

The racemization rates of the thalidomide enantiomers were studied in phosphate buffer (0.067 *M*, pH 7.4), plasma of various species and HSA solutions (4 g/100 ml). An aliquot of the standard solution of the enantiomers was evaporated to dryness under a stream of nitrogen. The final concentration of each enantiomer in the reaction mixture was from 9–18 n*M*. The reaction was started by adding 400 $\mu$l of the incubation medium and the mixture was stirred at 37°C in a water bath. At various times the reaction was stopped by immediate extraction as described below.

For inhibition experiments in HSA solutions, an aliquot of the capric acid sodium salt solution was evaporated to dryness under a stream of nitrogen and preincubated with HSA solution at 37°C for 15 min while stirring. The mixture was transferred to a tube containing thalidomide enantiomers and treated as described above.

*Extraction procedure*

For the determination of the enantiomeric ratio of thalidomide, 400 μl of the incubation mixtures were extracted twice with 2 ml of *n*-hexane–ethyl acetate (4:1 v/v) by shaking in a reciprocal shaker for 10 min. The organic layers were separated by centrifugation (2500 *g*, 10 min) and transferred to a tube; the extracts were combined and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl of dioxane and a 20-μl aliquot was injected onto the column.

For calibration, pure enantiomers of thalidomide were combined to obtain mixtures of known optical purity. Phosphate buffer (0.067 *M*, pH 7.4), human citric plasma and heparinized human plasma were spiked with these solutions prior to analysis.

*Estimation of the racemization half-lives*

The racemization of thalidomide enantiomers is presented as the change of enantiomeric excess (ee). The ee value is the enantiomeric excess expressed as a percentage $[(R - S/R + S) \times 100; (\%)]$ of the enantiomer initially present. The natural logarithm of ee is plotted *versus* linear time axis. The half-life of racemization can be calculated from the graph (pseudo-first order reaction).

For inhibition experiments with capric acid the ee is plotted *versus* the concentration of capric acid anion.

## RESULTS AND DISCUSSION

*Determination of the enantiomeric ratio*

The extraction of the different incubation media was carried out with a mixture of the non-polar solvent *n*-hexane and the polar solvent ethyl acetate. Samples with no interfering peaks were obtained. Owing to their anionic structure at pH 7.4, the products of hydrolysis of thalidomide were not extracted. The decrease of concentration of thalidomide by hydrolysis was ignored. The recovery of thalidomide after extraction was 68.4 ± 5.9% at the concentration of 2.2 μg/400 μl ($n = 7$).

For the calibration of the assay, mixtures of the enantiomers with a defined ratio were analysed at three different concentrations after extraction from various aqueous media (Table I). Linear calibration curves for both enantiomers were obtained.

*Racemization studies*

The racemization of the thalidomide enantiomers was studied in various aqueous media (phosphate buffer 0.067 *M*, pH 7.4, plasma of different species, HSA solutions). The racemization of the thalidomide enantiomers in phos-

TABLE I

CALIBRATION AND REPRODUCIBILITY OF THE DETERMINATION OF THALIDOMIDE ENANTIOMERS AFTER EXTRACTION

| Extraction medium | *R*-(+)-thalidomide (%) added | *R*-(+)-thalidomide (%) analysed (mean ± S.D., $n = 3$) |
|---|---|---|
| Phosphate buffer | 20 | 19.30 ± 0.99 |
| Phosphate buffer | 30 | 29.97 ± 1.36 |
| Phosphate buffer | 70 | 72.13 ± 0.54 |
| Human citric plasma | 20 | 19.55 ± 1.62 |
| Human citric plasma | 30 | 30.53 ± 0.96 |
| Human citric plasma | 70 | 71.07 ± 1.97 |
| Human citric plasma | 80 | 80.60 ± 2.62 |
| Heparinized human plasma | 30 | 29.04 ± 0.89 |
| Heparinized human plasma | 40 | 40.60 ± 2.19 |
| Heparinized human plasma | 60 | 60.15 ± 1.51 |
| Heparinized human plasma | 70 | 70.04 ± 1.40 |

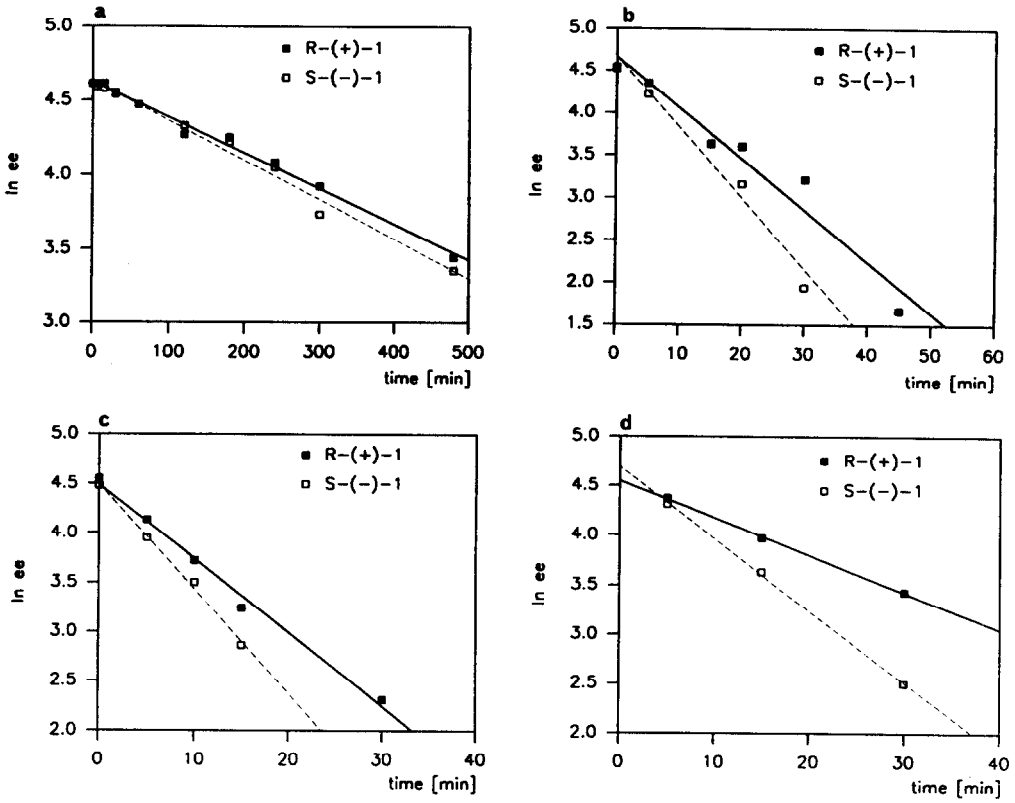


Fig. 2. Racemization of thalidomide (1) enantiomers in: (a) phosphate buffer pH 7.4, (b) human citric plasma, (c) rabbit citric plasma, (d) an essentially fatty acid-free albumin solution.

phate buffer 0.067 *M*, pH 7.4, at 37°C is presented in Fig. 2a. The half-lives of the racemization of the enantiomers are essentially the same within the limits of error (Table II), $t_{1/2} = 288.8$ min and $t_{1/2} = 260.5$ min for (+)- and (−)-thalidomide, respectively. Thus, as expected, the

TABLE II

THALIDOMIDE RACEMIZATION HALF-LIVES IN DIFFERENT INCUBATION MEDIA

| Incubation medium | Substrate | Half-life (min)[a] |
|---|---|---|
| Phosphate buffer | (+)-Thalidomide | 288.8 |
| Phosphate buffer | (−)-Thalidomide | 260.5 |
| Human citric plasma | (+)-Thalidomide | 11.5 |
| Human citric plasma | (−)-Thalidomide | 8.3 |
| Rabbit citric plasma | (+)-Thalidomide | 9.3 |
| Rabbit citric plasma | (−)-Thalidomide | 6.5 |
| Fatty acid-free albumin | (+)-Thalidomide | 18.5 |
| Fatty acid-free albumin | (−)-Thalidomide | 9.5 |
| Fraction V albumin | (+)-Thalidomide | 68.8 |
| Fraction V albumin | (−)-Thalidomide | 54.4 |

[a] The half-lives of racemization were calculated from the graphs of the racemization kinetics. The graphs were created by the averages of the measurements ($n = 3$–4) at each point of time.

spontaneous racemization rates of the enantiomers are identical.

Investigations of the rates of racemization in human (Fig. 2b) and rabbit (Fig. 2c) citric plasma showed a significant decrease in the half-lives (Table II); in human citric plasma $t_{1/2}$ was 11.5 min and 8.3 min, while in rabbit citric plasma $t_{1/2}$ was 9.3 min and 6.5 min for (+)- and (−)-thalidomide, respectively. There were no large differences between species, but (−)-thalidomide seems to racemize faster than (+)-thalidomide. Studies with heparinized plasma from both species demonstrated that the anticoagulant had no effect on racemization [4].

Further experiments had to clarify the cause of rapid racemization in plasma. Serum albumin is one of the main protein components of blood plasma. Therefore, the rate of racemization was studied in phosphate buffer in the presence of physiological concentrations of serum albumin. Fig. 2d shows the racemization kinetics in a solution of defatted human serum albumin. The half-lives of racemization in this incubation medium are similar to those in human plasma, 18.5 min and 9.5 min for (+) and (−)-thalidomide, respectively (Table II). The presence of albumin in the phosphate buffer drastically accelerates the racemization of the enantiomers. The rate of racemization was also investigated in a fraction V albumin solution. This albumin has a high content of fatty acids as a consequence of the preparation procedure. In contrast to the half-lives in defatted serum albumin, the half-lives of thalidomide racemization in a fraction V albumin solution were prolonged, 68.8 min for (+)-thalidomide and 54.4 min for (−)-thalidomide. Thus, the high content of fatty acids in this albumin seems to diminish the interaction of thalidomide with the albumin.

In order to investigate this interaction in further detail, inhibition experiments with capric acid anions in a defatted human serum albumin solution were carried out. Capric acid anions interact with an apolar binding site of human serum albumin. It has a strong inhibitory effect at this binding site, *i.e.* for the esterase-like activity of human serum albumin [7]. By adding increasing concentrations of this fatty acid anion the racemization of the thalidomide enantiomers was more and more suppressed (Fig. 3). Fig. 4

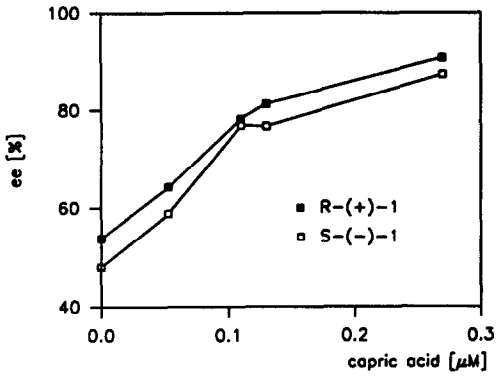


Fig. 3. Racemization of thalidomide (1) enantiomers in an essentially fatty acid-free albumin solution after preincubation with increasing concentrations of capric acid sodium salt (preincubation, 15 min; incubation, 15 min).

presents the inhibiting influence of capric acid anions on the racemization of the enantiomers in a defatted human serum albumin solution.

These results were supported by other experiments in our laboratory [4]. For metabolism studies, thalidomide enantiomers were incubated with rat liver microsomes. After 30 min incubation at 37°C, the racemization was very low (Fig. 5). The microsomal enzymes do not seem to

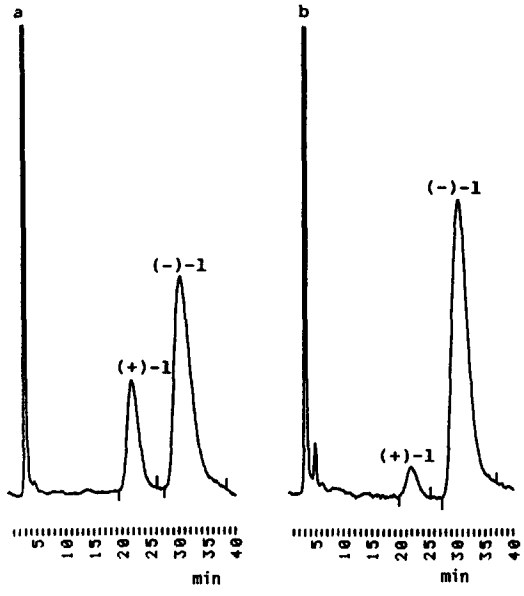


Fig. 4. High-performance liquid chromatogram of partially racemized (−)-thalidomide (1) extracted from an essentially fatty acid-free albumin solution without preincubation (a) and with preincubation of 0.27 $\mu M$ capric acid sodium salt (b). Chromatographic conditions: same as for Fig. 1b.

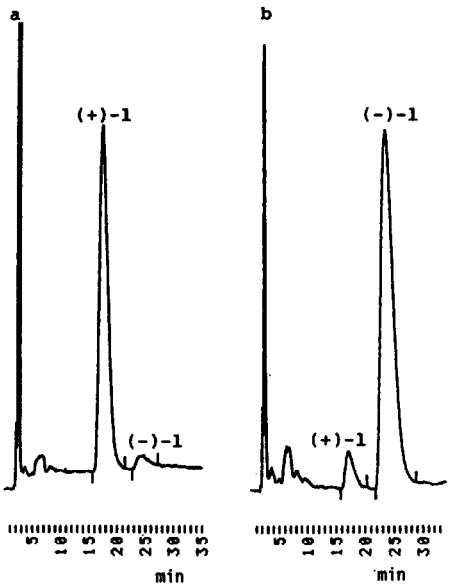


Fig. 5. High-performance liquid chromatogram of thalidomide (1) extracted from incubations with rat liver microsomes, *R*-(+)-thalidomide (a) and *S*-(−)-thalidomide (b). Chromatographic conditions: same as for Fig. 1b.

have the capability to racemize thalidomide enantiomers as quickly as human serum albumin.

## CONCLUSIONS

The investigations demonstrated the great influence of human serum albumin on the racemization of thalidomide enantiomers. This effect has also been observed for other chiral drugs [8]. On the other hand, liver microsomal enzymes do not have any influence on the racemization of thalidomide enantiomers. Thus, the enantioselectivity of thalidomide in relation to teratogenic effects [1] has to be re-evaluated.

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