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Investigation of the stereoselective in vitro biotransformation of glutethimide by high-performance liquid chromatography and capillary electrophoresis[1]

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Abstract

Due to our interest in drugs with a glutarimide structure, we reinvestigated the stereoselectivity of the in vitro biotransformation of the chiral hypnotic-sedative drug glutethimide. Glutethimide enantiomers were separated on a preparative scale by HPLC on cellulose tris(4-methylbenzoate) as chiral stationary phase. The enantiomeric purity was higher than 99%. A reversed-phase HPLC method was developed to determine the metabolites of glutethimide. After incubations with rat liver microsomes both enantiomers formed 5-hydroxyglutethimide as the main metabolite, as well as additional metabolites, of which some were formed stereoselectively. Mass spectrometry of the unknown metabolites indicated a hydroxylation in the ethyl side chain for two of the metabolites. A third metabolite was tentatively identified as desethylglutethimide.

Keywords: Enantiomer separation; Glutethimide; 5-Hydroxyglutethimide; Desethylglutethimide

1. Introduction

We have investigated the stereoselectivity of the in vitro biotransformation of the sedative drug glutethimide. Several studies of the in vivo metabolism of glutethimide have been described, but with very differing results. Keberle et al. [1] reported a substrate stereospecifity for the hydroxylation in experiments with dogs. Whereas *S*-(−)-glutethimide was hydroxylated in the ethyl side chain, *R*-(+)-glutethimide was hydroxylated at the glutarimide ring and formed exclusively 5-hydroxyglutethimide (Fig. 1). Kennedy and Fischer [2] could not confirm this stereospecificity for the formation of 5-hydroxyglutethimide in humans. They reported a stereoselectivity for the hydroxylation in the ethyl side chain: each enantiomer formed a different diastereomer of 1-hydroxyethylglutethimide.

Previously the enantioselectivity of the in vitro metabolism of the structurally similar sedative drug thalidomide was reported. Whereas *S*-(−)-thalidomide mainly formed 5-hydroxythalidomide, *R*-(+)-thalidomide preferentially formed two metab-

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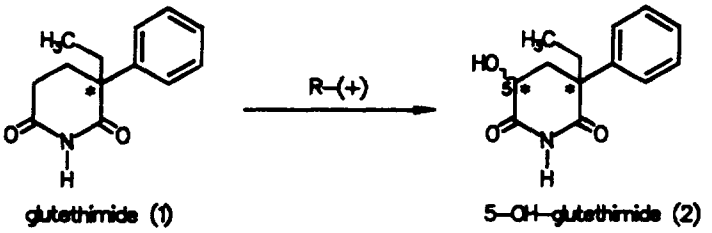

Fig. 1. Biotransformation of *R*-(+)-glutethimide.

olites, tentatively assigned as diastereomers of 5′-hydroxythalidomide (Fig. 2) [3,4].

The formation of 5-hydroxyglutethimide which has been reported by Keberle et al. [1] and Kennedy and Fischer [2] introduces a second chiral centre in the glutethimide molecule, thus leading, similar to thalidomide, to the possibility that diastereomers of 5-hydroxyglutethimide could be formed.

The aim of our study was to investigate the stereoselectivity of the biotransformation of glutethimide in vitro, concentrating on a possible formation of the diastereomers of 5-hydroxyglutethimide.

2. Experimental

2.1. Chemicals, reagents and buffer preparation

Racemic glutethimide (1) and racemic 5-hydroxyglutethimide (2) were kindly provided by Ciba-Geigy (Basel, Switzerland). Acetonitrile, ethyl acetate, methanol, *n*-hexane and 2-propanol were of HPLC quality and purchased from Baker (Gross-Gerau, Germany). Sodium dodecyl sulfate (SDS) was from Fluka (Fluka Chemie, Buchs, Switzerland). Analytical grade tetrabutylammonium hydrogensulfate (TBAH), H_3BO_3, NaOH, tris(hydroxymethyl)aminomethane, $MgCl_2$ and NADPH were purchased from Merck (Darmstadt, Germany), tris(hydroxymethyl)aminomethane hydrochloride was from Sigma (St. Louis, MO, USA) and phenobarbitone (Luminal) was from Bayer (Wuppertal, Germany).

Stock solutions of 100 m*M* H_3BO_3 were prepared in double distilled, deionized water and the pH was adjusted with 0.5 *M* NaOH. The run buffers were prepared accordingly after the addition of appropriate amounts of the surfactants. All solutions were filtered and degassed by sonication before use. Standard solutions of glutethimide and 5-hydroxyglutethimide were prepared in methanol. Aliquots of these standards were evaporated to dryness under a stream of nitrogen and reconstituted in the micelle-containing run buffer.

2.2. Apparatus

The system for the preparative separation consisted of a LC-8A preparative liquid chromatograph

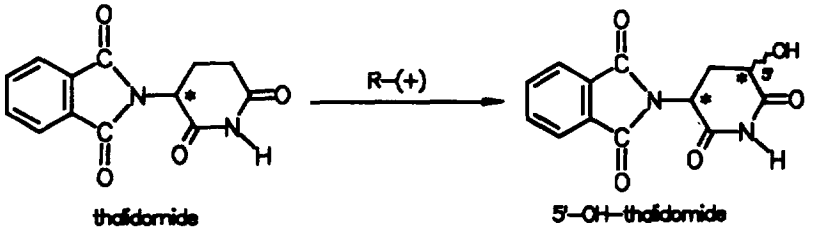

Fig. 2. Biotransformation of *R*-(+)-thalidomide.

(Shimadzu, Duisburg, Germany), a Rheodyne sample injector (Model 7125, Rheodyne) equipped with a 1.0-ml loop, a variable-wavelength detector (Knauer, Bad Homburg, Germany) and a D-2000 chromato-integrator (Merck-Hitachi, Darmstadt, Germany).

The HPLC system consisted of a Knauer HPLC pump 64, a Rheodyne sample injector (Model 7125) with a 20-μl loop, a variable-wavelength UV detector 655A (Merck-Hitachi) and a C-R3A Chromatopac integrator (Shimadzu).

The CE system consisted of a Beckman (Munich, Germany) P/ACE 2100 with an uncoated fused-silica capillary (Grom, Herrenberg, Germany) of 40 cm effective length×50 μm I.D. Prior to its use and between analyses the capillary was rinsed for 1 min with 0.1 *M* sodium hydroxide and run buffer. The measurements were performed with an applied voltage of 12.6 kV (field strength 268 V/cm), temperature 25°C. The average current was 0.015 mA. Samples were introduced by low-pressure injection for 5 s (about 5 nl).

The mass spectrometer was a Finnigan MAT 8430 (Finnigan MAT, Bremen, Germany).

2.3. Preparative separation

The separation of the glutethimide enantiomers on a preparative scale was achieved on a cellulose tris(4-methylbenzoate) column (20–45 μm) with methanol as the mobile phase. The flow-rate was 50 ml/min and the detector wavelength 257 nm. A 200-mg amount of racemic glutethimide was injected in 1 ml methanol. The enantiomers were collected and evaporated to dryness. Crystallization from acetone afforded the pure enantiomers. The purity of the enantiomers was determined by chiral HPLC on a Chiralcel OJ column. Each enantiomer was characterized by elemental analysis, mass spectrometry (70 eV, *m/z* (%) 217 (17) $[M]^+$, 189 (100) $[C_{11}H_{11}NO_2]^+$, 160 (31) $[C_{10}H_{10}NO]^+$, 132 (58) $[C_{10}H_{12}]^+$, 117 (86) $[C_9H_9]^+$), ^1H NMR, ^{13}C NMR and corresponded with the data for the racemate [5]. Moreover, the rotation data of $[\alpha]_D^{20}$ +186.17 for the *R*-(+)- and $[\alpha]_D^{20}$ −169.47 for the *S*-(−)-enantiomer were consistent with the literature [5,6].

2.4. Analytical resolution

The analytical resolution of glutethimide enantiomers was achieved by HPLC on a Chirasep DNBPG column (Merck, 250×4.6 mm I.D., particle size 5 μm) with *n*-hexane–2-propanol–acetonitrile (94.5:5.0:0.5, v/v) at a flow-rate of 0.6 ml/min, on a Chiralcel OD column (Daicel, Baker, 250×4.6 mm I.D., particle size 10 μm) with acetonitrile–water (35:65, v/v) at a flow-rate of 0.5 ml/min, and on a Chiralcel OJ column (Daicel, Baker, 250×4.6 mm I.D., particle size 10 μm) with methanol as the mobile phase at a flow-rate of 0.5 ml/min. The detection wavelength was 210 nm.

2.5. Achiral chromatography

The separation of glutethimide and its metabolite 5-hydroxyglutethimide was performed on a Superspher RP 8 (Merck, 250×4.6 mm I.D., particle size 4 μm) equipped with a RP 8 guard column (Merck, 4×4 mm, particle size 10 μm). The mobile phase was acetonitrile–0.01 *M* TBAH pH 6.5 (30:70, v/v) with a flow-rate of 0.7 ml/min. The detection wavelength was 210 nm.

2.6. In vitro biotransformation with liver microsomes

Microsomes were obtained from male Sprague–Dawley rats and a bovine as described [7]. The rats were pretreated with phenobarbitone for six days (50 mg/kg body weight). The protein concentration was determined according to Bradford [8]. The incubation mixture consisted of substrate (glutethimide enantiomers: about 6.14 μg), liver microsomes (equivalent to 2 mg protein) and cofactors (NADPH: about 0.5 μ*M*, $MgCl_2$ 6 μ*M*) in a total volume of 1.0 ml adjusted to pH 7.4 with 0.1 *M* tris buffer. Incubations were carried out under vigorous stirring in a water bath at 37°C for 15 min. The reaction was quenched by cooling to 0°C. The samples were extracted immediately with 3 ml ethyl acetate by shaking in a reciprocal shaker for 10 min. The tubes were centrifuged at 2500 *g* for 10 min. Subsequently the organic layer was transferred to a clean vial and evaporated to dryness under a stream of nitrogen.

The residue was either dissolved in the mobile phase of the achiral HPLC system or dissolved in the SDS-containing run buffer and analyzed.

2.7. Mass spectrometric experiments

The extracts of the microsomal incubations were separated by achiral HPLC. The fractions of the metabolites were collected after chromatography and subsequently reextracted from the mobile phase after addition of 1 ml water and 2 ml ethyl acetate (10 min shaking, 10 min centrifugation at 2500 *g*). The organic layer was evaporated to dryness under a stream of nitrogen. The residue was subjected to mass spectrometry by chemical ionization (CI) with ammonia and by electron impact ionization (EI, 70 eV).

3. Results and discussion

3.1. Chiral resolution

Racemic glutethimide (1) was separated on cellulose tris(4-methylbenzoate) on a preparative scale [5]. The elution order was *R*-(+)- before *S*-(−)-glutethimide.

The results of the analytical resolutions are summarized in Table 1. A simultaneous separation of glutethimide (1) and 5-hydroxyglutethimide (2) could be obtained on the Chiralcel OD and Chiralcel OJ column (Fig. 3). On the Chirasep DNBPG and on the Chiralcel OD column the elution order was *S*-(−)- before *R*-(+)-glutethimide. The elution order was reversed by employing the Chiralcel OJ column. The enantiomeric purity was determined on a Chiralcel OJ column, which yielded the best enantioseparation, and exceeded 99%.

Table 1
Enantioseparation of racemic glutethimide and 5-hydroxyglutethimide on different chiral columns

Column	Glutethimide		5-Hydroxyglutethimide	
	α	R	α	R
Chirasep DNBPG	1.10	0.89	n.t.[a]	n.t.[a]
Chiralcel OD	1.21	1.33	1.14	1.00
Chiralcel OJ	1.66	3.85	1.47	3.50

[a] n.t. = not tested.

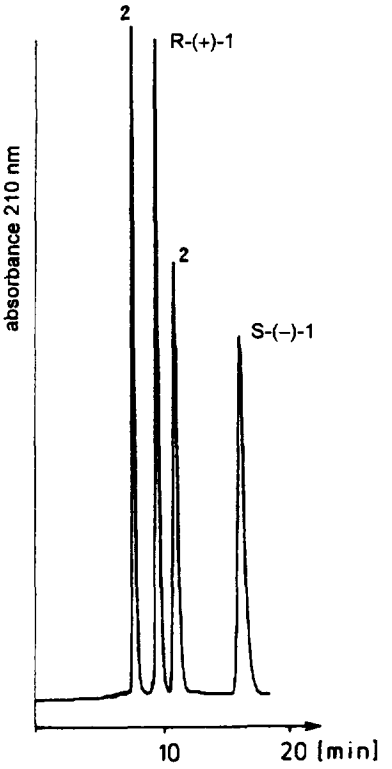

Fig. 3. Separation of glutethimide (1) and 5-hydroxyglutethimide (2) enantiomers. Chiralcel OJ column; chromatographic conditions: methanol, 0.5 ml/min; 210 nm (UV).

3.2. In vitro metabolism

The biotransformation of the enantiomers was studied in vitro using rat and bovine liver homogenates. The extraction of the incubation medium yielded a recovery of about 91% for glutethimide and 86% for 5-hydroxyglutethimide.

In incubations with rat liver microsomes glutethimide (1) was extensively metabolized. *R*-(+)-Glutethimide formed five major metabolites (known 5-hydroxy-glutethimide (2) and unknown metabolites subsequently described as A–D) and several minor metabolites (Fig. 4a). Incubations of *S*-(−)-glutethimide yielded the same metabolites with the exception that metabolite A, which appears

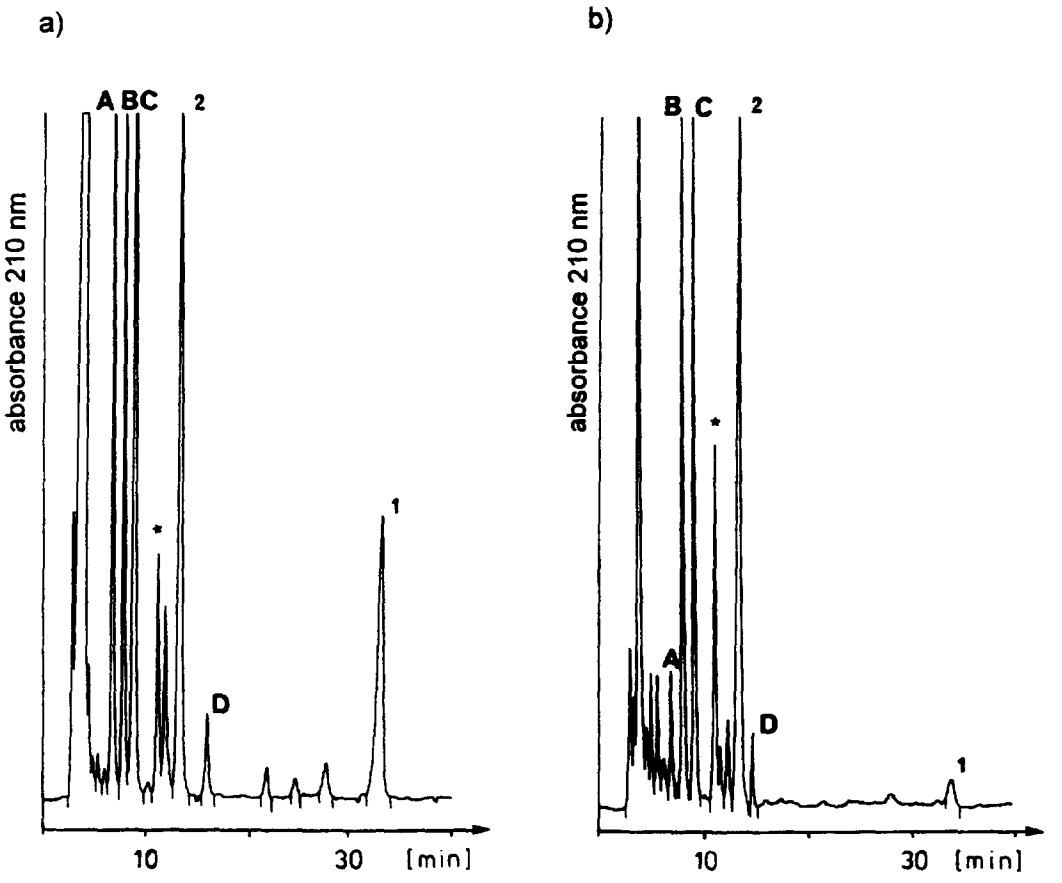

Fig. 4. Chromatograms of (a) *R*-(+)- and (b) *S*-(−)-glutethimide extracted from incubations with rat liver microsomes. The asterisk indicates endogenous substance from the liver preparation. Chromatographic conditions: Superspher RP 8, RP 8 guard column, acetonitrile–0.01 *M* TBAH pH 6.5 (30:70, v/v), 0.7 ml/min, 210 nm (UV).

to be stereoselectively formed from *R*-(+)-glutethimide, occurs only in minor amounts (Fig. 4b). 5-Hydroxyglutethimide (2), the major metabolite, was formed from both enantiomers in equal proportions.

CE was employed as a second analytical technique to confirm these results obtained by HPLC due to the high separation power of this technique. The stereoselectivity of the in vitro biotransformation of glutethimide could be confirmed: incubations of *R*-(+)-glutethimide yielded the metabolites A–D and 5-hydroxyglutethimide (2) (Fig. 5a), whereas incubations of *S*-(−)-glutethimide yielded the metabolites B–D, as well as 5-hydroxyglutethimide, but very low quantities of metabolite A (Fig. 5b).

Upon incubation of *R*-(+)-glutethimide with bovine liver microsomes the metabolites A, B, C and 5-hydroxyglutethimide (2) were formed (Fig. 6a). In contrast to rat liver microsomes, metabolite D could not be detected, but another metabolite E, which eluted before 5-hydroxyglutethimide, was formed in higher rates. The major metabolite was C. The main difference in the incubations with *S*-(−)-glutethimide was the low quantity of metabolite B (Fig. 6b), which appears to be stereoselectively formed from *R*-(+)-glutethimide.

These metabolism experiments depended on the presence of NADPH in the incubation mixture. The metabolism was inhibited by adding metyrapone, a cytochrome P450 inhibitor [5]. The most likely

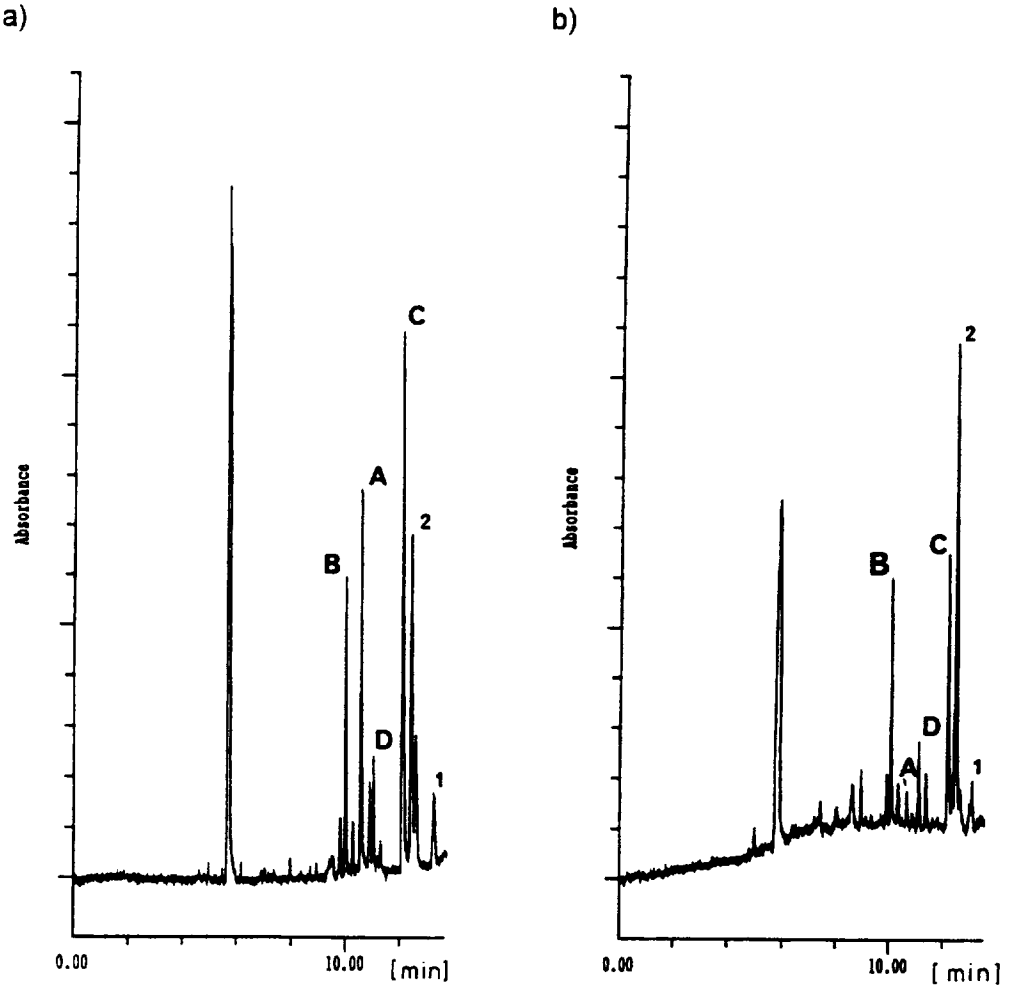

Fig. 5. Electropherograms of (a) *R*-(+)- and (b) *S*-(−)-glutethimide extracted from incubations with rat liver microsomes. Conditions: 100 m*M* SDS, 100 m*M* borate electrolyte pH 7.0, 268 V/cm, 210 nm (UV).

biotransformation step is thus hydroxylation of glutethimide.

3.3. Mass spectrometric experiments

In order to confirm its structure, mass spectrometric experiments were carried out with 5-hydroxyglutethimide and with the fractions of the isolated metabolites A–D.

The mass spectrum of the fraction of 5-hydroxyglutethimide corresponded with the mass spectrum of the reference, confirming the structure of this metabolite.

Direct chemical ionization with ammonia (DCI) yielded for both metabolites A and B a molecular peak at m/z 234 $[M+H]^+$ (Fig. 7a).

These molecular peaks were 16 mass units higher than the molecular peak of glutethimide under the same conditions. An insertion of oxygen may have occurred.

Additional experiments were carried out after electron impact ionization (EI) of the metabolites A and B in order to obtain information about the position of the hydroxylation, due to the intensive fragmentation in this technique. An insertion of oxygen could have occurred at the glutarimide ring,

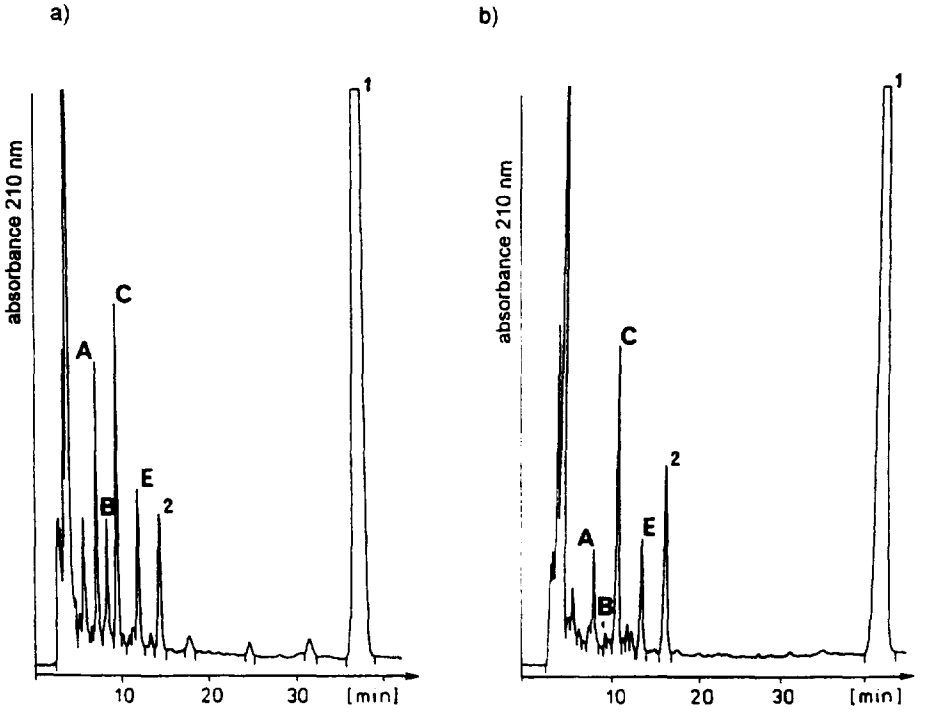

Fig. 6. Chromatograms of (a) *R*-(+)- and (b) *S*-(−)-glutethimide extracted from incubations with bovine liver microsomes. Chromatographic conditions: see Fig. 4.

at the ethyl side chain or at the aromatic ring. Mass spectra which were obtained of glutethimide and of 5-hydroxyglutethimide after EI indicated that the main fragmentation step always was a cleavage of the ethyl side chain. This fragmentation resulted for glutethimide in a peak at *m/z* 189 (desethylglutethimide) (Fig. 8a), whereas 5-hydroxyglutethimide formed a peak at *m/z* 205 (Fig. 8b), which was 16 mass units higher, due to the additional oxygen. The mass spectra of the metabolites A and B showed both a peak at *m/z* 189 (Fig. 8c).

This excludes a hydroxylation of glutethimide at the glutarimide as well as at the aromatic ring. Thus metabolites A and B could be isomers of hydroxyethyl-glutethimide.

Using DCI for metabolite C molecular peaks at *m/z* 190 and *m/z* 207 with 28 mass units lower than glutethimide were detected (Fig. 7b). Metabolite C is very likely desethylglutethimide, due to the fragmentation of the ethyl side chain (28 mass units).

Unlike thalidomide the in vitro biotransformation of glutethimide did not lead to a stereoselective hydroxylation at position 5 of the glutarimide ring with formation of both diastereomers of 5-hydroxyglutethimide. The possible formation of a second diastereomer cannot absolutely be excluded, because the metabolites which were formed in lower concentrations could not be analyzed by mass spectrometric experiments.

4. Conclusion

The biotransformation of glutethimide was studied in vitro by incubation with rat and bovine liver microsomes. *R*-(+)-Glutethimide formed five major metabolites (A–D) and 5-hydroxyglutethimide; *S*-(−)-glutethimide formed the same metabolites. However, from this enantiomer metabolite A was only formed in low quantities with rat liver microsomes and metabolite B with bovine liver microsomes. No stereoselectivity could be observed for the formation of 5-hydroxyglutethimide. Moreover, as shown by

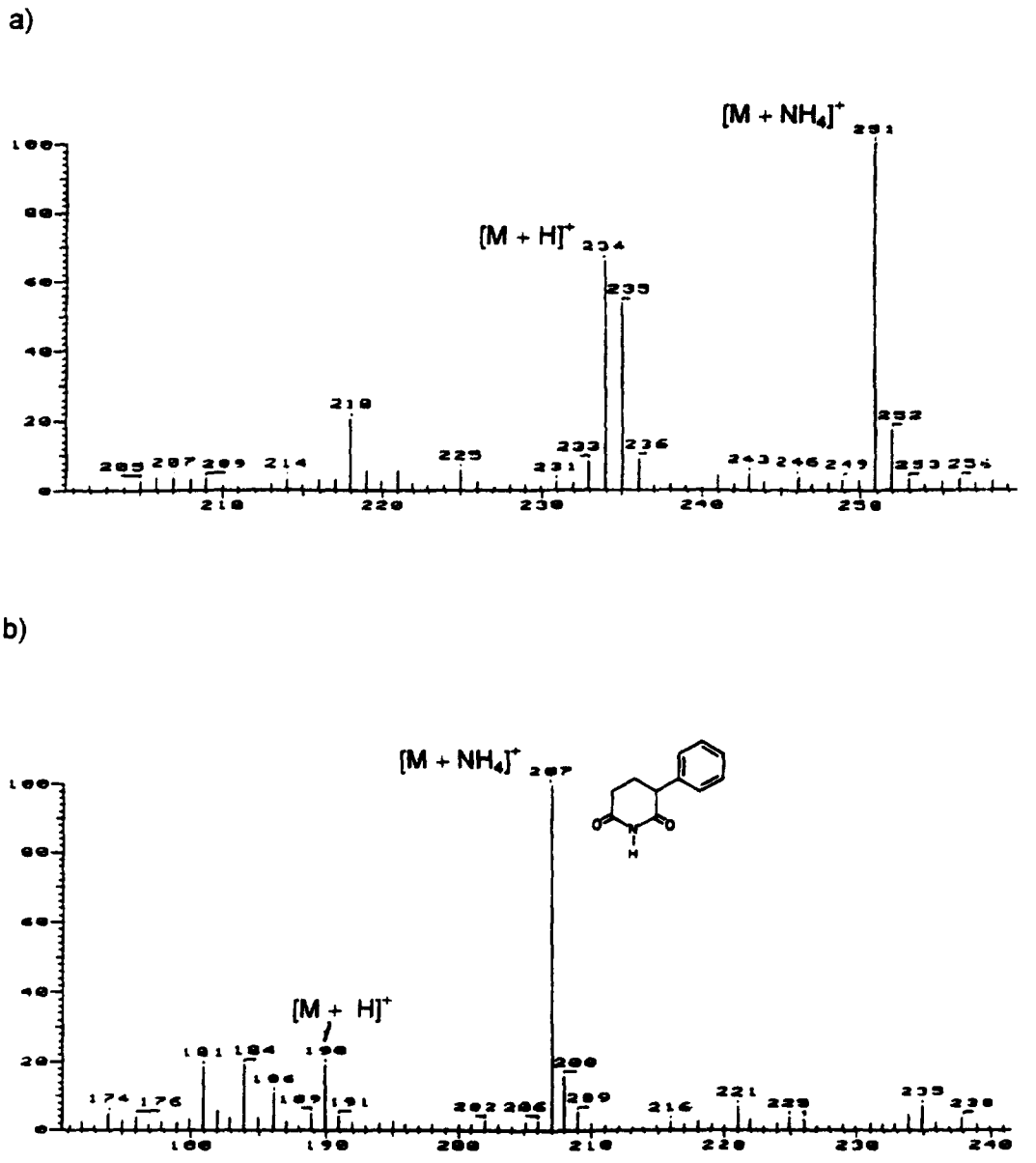

Fig. 7. Mass spectrum of the fraction of (a) metabolite A and (b) metabolite C obtained after direct chemical ionization (DCI). (a) *m/z* 218 and *m/z* 235 resulted from contamination with glutethimide.

mass spectrometry only one diastereomer of 5-hydroxyglutethimide was formed in major quantities. Metabolites A and B were tentatively assigned as isomers of hydroxyethyl-glutethimide, metabolite C as desethylglutethimide.

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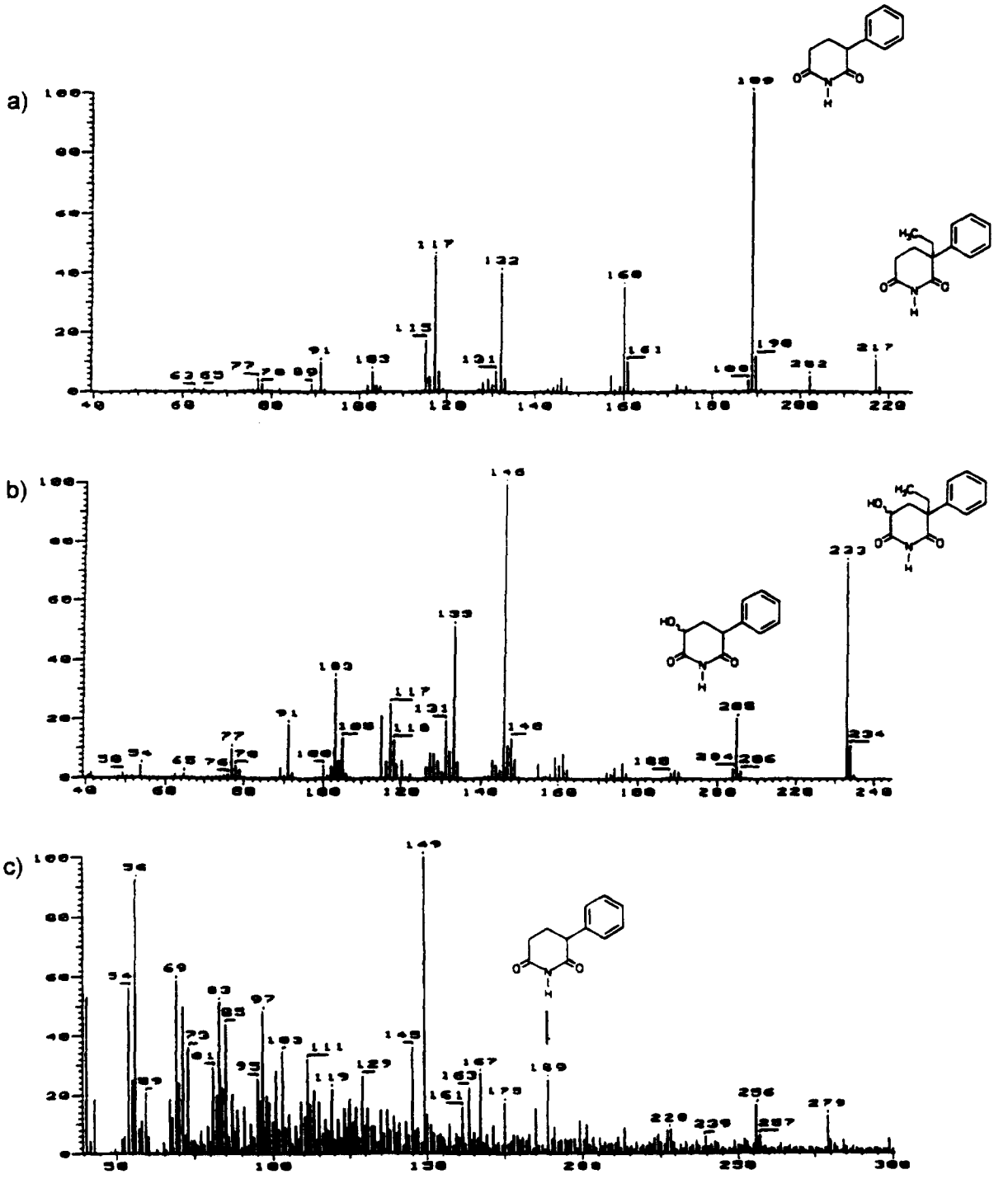

Fig. 8. Mass spectrum of (a) glutethimide, (b) 5-hydroxyglutethimide and (c) the fraction of metabolite A obtained after electron impact ionization (EI). (c) m/z 149, 167, 279 resulted from contamination with dioctylphthalate.

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