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Investigation of the in vitro biotransformation and simultaneous enantioselective separation of thalidomide and its neutral metabolites by capillary electrophoresis

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Abstract

Reversed-phase liquid chromatography is a long established method for the analysis of drug metabolism. The current investigation demonstrates that micellar electrokinetic capillary chromatography can be an attractive alternative. Two methods were developed using sodium dodecyl sulfate and hexadecyltrimethylammonium bromide for the determination of possible hydroxylated metabolites of the former sedative drug thalidomide (Contergan) in order to study the in vitro metabolism of the drug by incubation with rat liver microsomes. The biotransformation was found to be stereoselective: *S*-(−)-thalidomide mainly formed 5-hydroxythalidomide, whereas *R*-(+)-thalidomide was preferentially transformed to two metabolites tentatively assigned to be diastereomers of 5′-hydroxythalidomide.

Furthermore, the simultaneous enantioseparation of thalidomide and two of its possible hydroxylated metabolites was achieved using capillary electrophoresis with negatively charged carboxymethyl-β-cyclodextrin. The dependencies of the selectivity of the enantioseparation on the concentration of the chiral additive and the pH of the run buffer were investigated.

1. Introduction

Capillary electrophoresis (CE) has been originally developed as a microanalytical technique for the analysis of ionogenic compounds [1]. Later, micellar electrokinetic capillary chromatography (MECC) allowed the analysis of neutral compounds [2,3]. In 1989 enantioseparations of charged racemates were reported using cyclodextrins (CD) as chiral selectors in the free solution mode [4]. The separation of some basic drugs and their metabolites and the resolution of enantiomers in the same run using CE is described [5]. The resolution of neutral drug enantiomers is also possible by using charged chiral selectors in CE [6,7].

We are investigating the biotransformation of the former sedative drug thalidomide (*rac*-1,3-dioxo-2-[2′,6′-dioxopiperidin-3′-yl]-isoindole) (Contergan) (Fig. 1) which was withdrawn from the market because of its teratogenic effects [8,9]. More than 30 years later the cause of the teratogenic effects is still not known, although there are a number of hypotheses discussing the cause. Yet for some time thalidomide has been

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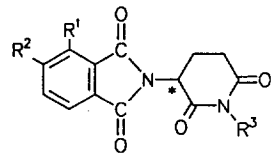

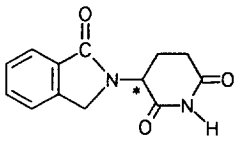

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		R^1	R^2	R^3
thalidomide	1	H	H	H
4-OH-thalidomide	2	OH	H	H
5-OH-thalidomide	3	H	OH	H
N-OH-thalidomide	4	H	H	OH

Fig. 1. Structure of the racemic drugs.

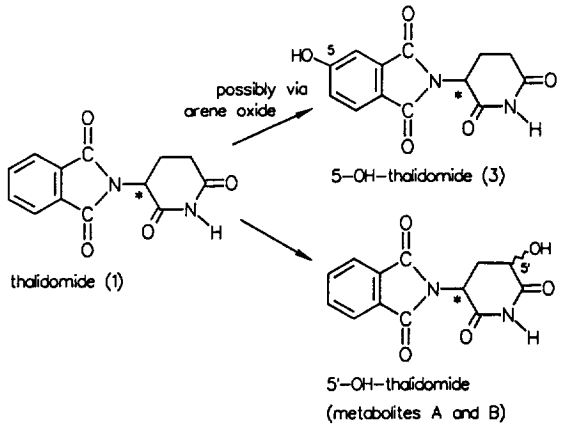

Fig. 2. Proposed biotransformation of the enantiomers of thalidomide.

making a clinical comeback. The renewed interest in thalidomide is based on its anti-inflammatory activity for the treatment of leprosy [10] and on the recently discovered inhibition of the HIV-1-virus [11]. The subsequent possible application of the drug to a broader collective of patients emphasizes the importance to clarify the cause of the teratogenic effects of thalidomide. Metabolic activation to reactive arene oxides, which are very unstable and can be detoxified to less reactive phenols is one theory which is discussed as a possible cause of teratogenic effects [12].

Previous studies [13] performed by high-performance liquid chromatography (HPLC) showed that the drug despite slow racemisation [14] undergoes a stereoselective biotransformation in vitro: *S*-(−)-thalidomide mainly formed 5-hydroxythalidomide. *R*-(+)-thalidomide was transformed to two new metabolites, A and B, which were tentatively identified as the diastereomers of 5'-hydroxythalidomide (Fig. 2).

The present study was conducted in order to develop a MECC method for the separation of thalidomide and its possible hydroxylated phase I metabolites. With the help of this method the results of the in vitro biotransformation obtained by HPLC should be verified.

Furthermore, for the first time the separation of the neutral thalidomide and its neutral metabolites and enantiomeric resolutions in the same run using charged carboxymethyl-β-CD (CM-β-CD) [6] as a buffer additive is described.

2. Experimental

2.1. Chemicals and reagents

Racemic thalidomide (1) and EM 12 (5) were kindly provided by 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 [14]. The purity of the enantiomers was determined by chiral HPLC and exceeded 99.5%. 4-Hydroxythalidomide (2), 5-hydroxythalidomide (3), and N-hydroxythalidomide (4) were synthesized and characterized in our laboratory [15].

Sodium dodecyl sulfate (SDS) and hexadecyltrimethylammonium bromide (HTAB) were from Fluka (Fluka Chemie, Buchs, Switzerland). CM-β-CD (substitution degree approx. 2.1) was a gift from Wacker Chemie (München, Germany).

Analytical grade KH_2PO_4, H_3BO_3, H_3PO_4, NaOH, tris(hydroxymethyl)aminomethane, $MgCl_2$, and NADPH were purchased from Merck (Darmstadt, Germany). Analytical grade

tris(hydroxymethyl)aminomethane hydrochloride was from Sigma (St. Louis, MO, USA).

2.2. Apparatus

Achiral separations were performed on a Beckman (Munich, Germany) P/ACE 2100 and a P/ACE 5510 capillary electrophoresis system 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) for the SDS-containing electrolyte and 10.0 kV (field strength 213 V/cm) for the HTAB-containing electrolyte, temperature 25°C. The average current was 0.015 mA. Samples were introduced by low-pressure injection for 5 s (about 5 nl). Detection was carried out at 214 and 230 nm.

Chiral separations were performed on a Grom capillary electrophoresis system 100, equipped with a Linear Instruments (Reno, NV, USA) UVIS 200 detector and a HP 3396A integrator (Hewlett-Packard, Avondale, PA, USA) with an uncoated fused-silica capillary (Grom) of 40 cm effective length × 50 μm I.D. The measurements were performed with an applied voltage of 24.0 kV (field strength 400 V/cm), temperature 21°C. The average current at the beginning of a run was 0.055 mA; it decreased during the run to 0.033 mA. Prior to its use and between analyses the capillary was rinsed with 100 μl 0.1 *M* sodium hydroxide and 100 μl run buffer. The racemic samples were introduced hydrostatically (10 cm) for 10 s at the anodic end (about 10 nl). The detection of the solutes was carried out at 225 nm. The anode and the cathode buffers had the same pH and molarity as the run buffer, but contained no chiral selector.

2.3. Buffer and sample preparation

Stock solutions of 100/50 m*M* H_3BO_3 were prepared in double distilled, deionized water and the pH was adjusted with 0.5 *M* NaOH. Stock solutions of 50 m*M* KH_2PO_4 were as well prepared in double distilled, deionized water. The pH value of 4.4 was adjusted with 0.5 *M* H_3PO_4 and the pH values 5.0 and 6.0 with 0.5 *M* NaOH.

The run buffers were prepared accordingly after the addition of appropriate amounts of the micelle-forming surfactants or the chiral selector. All solutions were filtered and degassed by sonication before use.

Standard solutions of the racemic drugs were prepared in acetonitrile for the achiral separations. Aliquots of these standards were evaporated to dryness under a stream of nitrogen and reconstituted in the micelle-containing electrolyte.

For the enantioseparation experiments the racemic drugs were injected as a solution in methanol–50 m*M* phosphate buffer (ratio 1:3).

2.4. In vitro biotransformation with liver microsomes

Microsomes were obtained from male Sprague–Dawley rats with pretreatment with phenobarbital for 6 days (50 mg/kg body weight). After fasting for 24 h, the homogenates were prepared according to a published method [16]. The protein concentration was determined as described by Bradford [17]. The incubation mixture consisted of substrate (thalidomide enantiomers about 5 μg), liver microsomes (equivalent to 1 mg protein) and cofactors (NADPH about 2 μ*M*, $MgCl_2$ 6 μ*M*). The total volume was 1.0 ml adjusted with Tris buffer 0.1 *M*, pH 7.4, 37°C. Incubations were carried out with stirring in a water bath at 37°C for 30 min. The reaction was stopped by cooling to 0°C and immediate extraction. The extraction was performed 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 tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 10 μl of the SDS-containing electrolyte and analyzed.

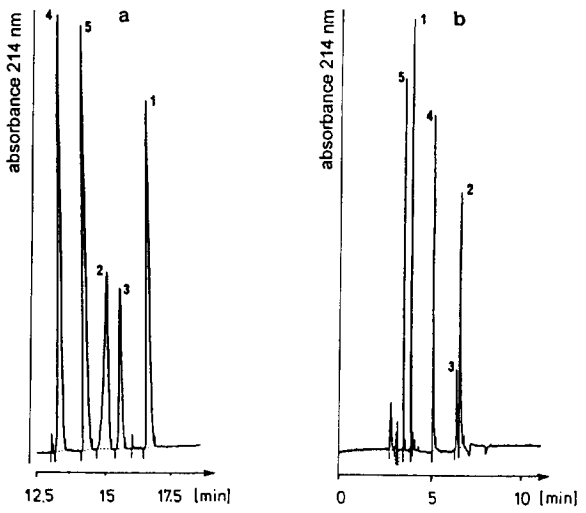

Fig. 3. MECC separation of thalidomide (1), of possible hydroxylated metabolites (2–4) and of EM 12 (5). Conditions: (a) 100 m*M* SDS, 100 m*M* borate electrolyte pH 7.0, 268 V/cm, detection 214 nm; (b) 20 m*M* HTAB, 50 m*M* borate electrolyte pH 7.0, 213 V/cm, detection 214 nm.

3. Results and discussion

3.1. In vitro metabolism

The structures of thalidomide (1), its possible hydroxylated phase I metabolites 4-hydroxythalidomide (2), 5-hydroxythalidomide (3) [13,18], N-hydroxythalidomide (4), and the thalidomide analogue EM 12 (5) are presented in Fig. 1. All compounds could be resolved by using SDS as an anionic surfactant (Fig. 3a). HTAB as a cationic surfactant caused a reversal of the electroosmotic flow. The separation is shown in Fig. 3b [19]. 4-Hydroxythalidomide (2) and 5-hydroxythalidomide (3) could only be partially resolved.

The metabolism of the thalidomide enantiomers was studied in vitro with rat liver microsomes. The stereoselectivity of the in vitro biotransformation of thalidomide is supported by these results. In Fig. 4, typical MECC electro-

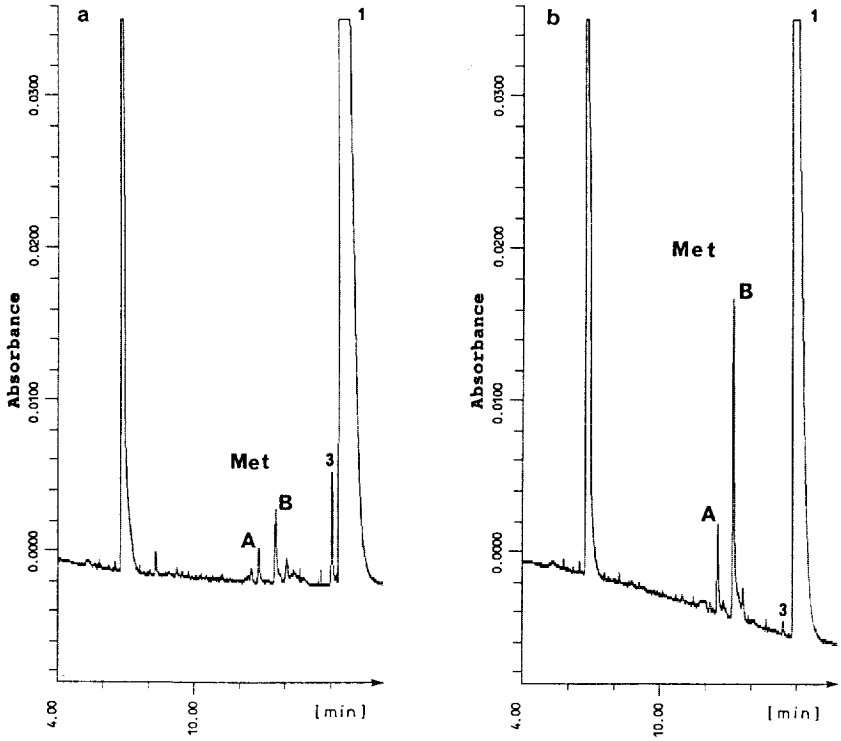

Fig. 4. MECC separation of *S*-(−)-thalidomide (a) and *R*-(+)-thalidomide (b), extracted from incubations with rat liver microsomes. Conditions: see Fig. 3a, detection 230 nm.

pherograms of the incubation of *R*-(+)- and *S*-(−)-thalidomide with rat liver microsomes are presented. As found previously in our laboratory [13] *S*-(−)-thalidomide mainly formed 5-hydroxythalidomide [18], whereas *R*-(+)-thalidomide was transformed to the two new metabolites A and B, which were tentatively identified as the diastereomers of 5′-hydroxythalidomide [13].

3.2. Simultaneous enantioselective separation of thalidomide and metabolites

Thalidomide and its metabolites cannot be separated or resolved using uncharged cyclodextrins, whereas separations could be achieved using negatively charged CM-β-CD. The results are summarized in Table 1. α is calculated as the ratio of t_2 and t_1, which are the migration times of the second and the first eluted enantiomers, respectively. The resolution of the enantiomers was calculated according the following equation:

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2}$$

where t_1 and t_2 are the migration times and w_1 and w_2 are the baseline peak widths of the first and second eluted enantiomers, respectively.

Thalidomide (1), 4-hydroxythalidomide (2), and 5-hydroxythalidomide (3) could be separated at pH values 4.4/5.0/6.0 with a concentration of 15 m*M* CM-β-CD. For N-hydroxythalidomide (4) only a poor resolution could be achieved at pH value 4.4.

In the tested pH range 4.4/5.0/6.0 the carboxylic function is deprotonated. Thus the CD obtains an own mobility and acts as a mobile pseudophase. This allows the simultaneous separation and chiral resolution of thalidomide (1), 4-hydroxythalidomide (2) and 5-hydroxythalidomide (3) without addition of another micelle-forming surfactant at pH 6.0 (pH above the pK_a value of the CM-β-CD) (Fig. 5). At this pH value a simultaneous separation and the

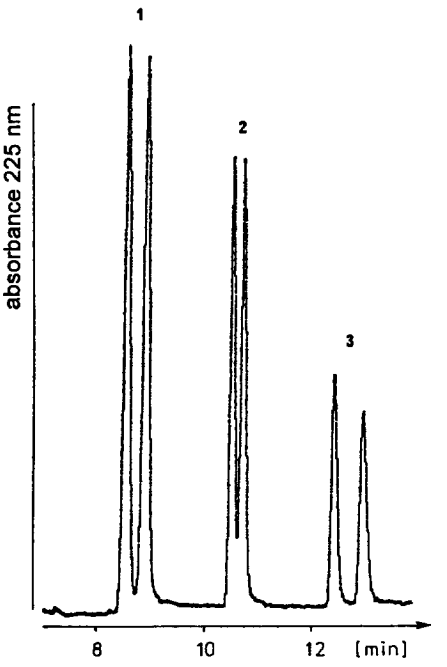

Fig. 5. Separation of thalidomide (1), 4-hydroxythalidomide (2), 5-hydroxythalidomide (3) enantiomers. Conditions: 50 m*M* phosphate buffer pH 6.0, 15 m*M* CM-β-CD, 400 V/cm, detection 225 nm.

Table 1
Chiral separation of the racemic drugs at different pH values

	pH 6.0		pH 5.0		pH 4.4	
	α	R_s	α	R_s	α	R_s
Thalidomide	1.054	3.49	1.046	1.83	1.049	2.36
4-Hydroxythalidomide	1.026	1.48	1.023	1.26	1.031	2.05
5-Hydroxythalidomide	1.073	3.28	1.058	3.65	1.070	3.97
N-Hydroxythalidomide	no separation		no separation		1.011	1.13

Conditions: 15 m*M* CM-β-CD, 50 m*M* phosphate electrolyte pH 6.0/5.0/4.4.

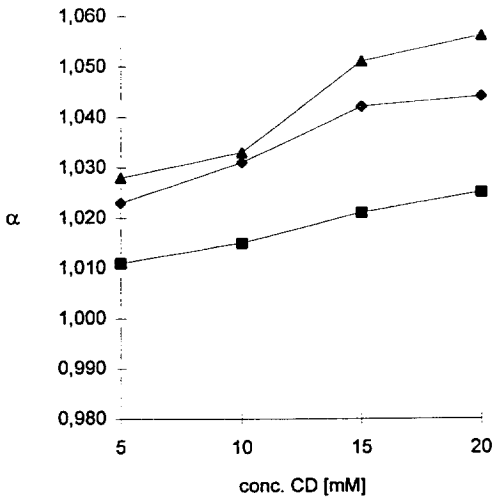

Fig. 6. Plot of the separation factors of thalidomide (◆), 4-hydroxythalidomide (■), and 5-hydroxythalidomide (▲) enantiomers vs. the concentration of CM-β-CD in the run buffer. Conditions: 50 m*M* phosphate buffer pH 6.0, 400 V/cm, detection 225 nm.

chiral resolution were possible in a concentration range of 5–20 m*M* CM-β-CD, the separation factor increasing with the CD concentration (Fig. 6).

4. Conclusion

MECC allows the separation of hydroxylated possible phase I metabolites of thalidomide, representing an attractive alternative to HPLC for the analysis of drug metabolism. The highly stereoselective biotransformation of thalidomide enantiomers with rat liver microsomes [13] was supported.

Furthermore, it is demonstrated that the negatively charged CM-β-CD is a useful chiral additive in CE, as far as it enables not only chemical selectivity but as well enantioselectivity. Therefore, CM-β-CD allows the simultaneous separation and enantioseparation of neutral drugs and their neutral metabolites.

References

[1] J.W. Jorgenson and K.D. Lukacs, Anal. Chem., 53 (1981) 1298.
[2] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, Anal. Chem., 56 (1984) 111.
[3] S. Terabe, K. Otsuka and T. Ando, Anal. Chem., 57 (1985) 834.
[4] S. Fanali, J. Chromatogr., 474 (1989) 441.
[5] M. Heuermann and G. Blaschke, J. Chromatogr., 648 (1993) 267.
[6] T. Schmitt and H. Engelhardt, Chromatographia, 37 (1993) 475.
[7] B. Chankvetadze, G. Endresz and G. Blaschke, Electrophoresis, 15 (1994) 804.
[8] W. Lenz, Dtsch. Med. Wochenschr., 86 (1961) 2555.
[9] W. Lenz and K. Knapp, Dtsch. Med. Wochenschr., 87 (1962) 1232.
[10] R.L. Barnhill and A.C. Mc Dougall, J. Am. Acad. Dermatol., 7 (1982) 317.
[11] G. Kaplan, Proc. Natl. Acad. Sci. USA, 90 (1993) 5974.
[12] H. Koch, Sci. Pharm., 49 (1981) 67.
[13] B. Knoche and G. Blaschke, Chirality, 6 (1994) 221.
[14] B. Knoche and G. Blaschke, J. Chromatogr. A, 666 (1994) 235.
[15] H.R. Hess, Ph.D. Thesis, University of Münster, Münster, 1990.
[16] F. Westhoff, Ph.D. Thesis, University of Münster, Münster, 1991.
[17] M. Bradford, Anal. Biochem., 72 (1976) 248.
[18] C.A. Lisek, Dissertation, Johns Hopkins University, Baltimore, MD, 1986, Uni Dissertation Information Service No. 8609337, Ann Arbor, MI, 1988.
[19] C. Weinz and G. Blaschke, presented at the 6th International Symposium on High-Performance Capillary Electrophoresis, San Diego, CA, January 31–February 3, 1994, poster P-623.