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Recent results of biotransformation of drugs: investigation of the in vitro biotransformation of thalidomide using a dual cyclodextrin system in capillary electrophoresis

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Abstract

A previously developed capillary electrophoresis method for the simultaneous separation and enantioseparation of thalidomide (TD) and its hydroxylated metabolites was extended to one additional biotransformation product. The dual chiral selector system using native β-cyclodextrin (β-CD) and the negatively charged sulfobutyl-β-CD (SBE-β-CD) was slightly modified up to a concentration of 12 mg/ml running buffer of each CD. The carrier mode in which these buffer additives transport the neutral compounds to the detector as well as the use of a polyacrylamide-coated capillary were necessary to achieve reproducible enantioseparations of all eight analytes.

Keywords: Biotransformation; Thalidomide; Dual cyclodextrin system; Capillary electrophoresis

1. Introduction

The former sedative-hypnotic drug thalidomide (TD) was introduced in the late 1950s under the trade name Contergan® but reports linking the use of TD to an increase of birth defects led to the removal of the drug from the general distribution. Although TD was identified to be a human teratogen [1,2], it has found a new therapeutic license [3] due to its anti-inflammatory and immunomodulating properties. The drug can be used for the treatment of leprosy [4], graft-versus-host disease following bone marrow transplantation, rheumatoid arthritis and several dermatological diseases [5,6]. In 1961, Lenz [1] and McBride [2] independently associated the use of TD by mothers early in the pregnancy with the occurrence of phocomelia, but the mechanism of teratogenesis remains unknown. Recently, Wells and et al. demonstrated the bioactivation of TD by embryonic prostaglandin H synthase (PHS) which causes oxidative damage to the DNA [7]. The involvement of the free radical-initiated reactive oxygen species (ROS) which resulted in DNA oxidation can be reduced or abolished by pretreatment with the free radical spin trapping agent alpha-phenyl-*N*-*t*-butyl-nitrone (PBN). Furthermore, the irreversible PHS inhibitor acetylsalicylic acid was found to be embryoprotective and to decrease the TD-initiated fetal limb anomalies [8]. On the other hand, evidence that metabolite formation is mediated by cytochrome P-450 is presented in an in vitro lymphocyte assay system [9] and in hepatic microsomes [10]. Three well-established P-450 inhibitors, metyrapone, SKF 525A and *N*-octylamine were applied in vitro and prevented TD activation. As both hypotheses remain tenable, we have focussed our work on the cytochrome P-450 catalyzed biotransformation.

2. Materials and methods

2.1. Capillary electrophoresis

The HP^{3D} capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with an diode-array detector was used for the CE studies. Chiral separations were performed in capillaries of 50 cm effective length and 50 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) which were previously coated

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by polyacrylamide as described in Ref. [11]. β-Cyclodextrin (β-CD) (12 mg/ml) and SBE(4)-β-CD (12 mg/ml) as chiral selectors were dissolved in 5 mM ammonium acetate (BGE) at pH 4.5. The measurements were carried out with an applied voltage of 30 kV (reversed-polarity) and the average current was 20 μA. Samples were injected by low pressure (50 mbar) for 7 s and detected at 230 nm. Between the analyses the capillary was rinsed for 2 min with methanol and for another 2 min with running buffer.

2.2. Preparation of liver fractions and in vitro biotransformation by hepatic microsomes

Hepatic subcellular fractions were prepared from male Sprague–Dawley rats that had been pretreated with 50 mg/kg body weight phenobarbital for 6 days. Livers were homogenized in buffer (25 mM sucrose/5 mM Tris–HCl/0.5 mM EDTA) at 4 °C. Supernatant ($10\,000 \times g$) and microsomal fractions ($100\,000 \times g$) were prepared according to a method described in Ref. [12] and stored at −80 °C.

The incubation mixture with a total volume of 1 ml consisted of substrate (standard solutions of racemic TD [200 μl] and its enantiomers [100 μl], evaporated to dryness), 410 μl 0.1 M Tris–buffer (pH 7.4 at 37 °C), 245 μl NADPH-solution (8 mg/ml buffer), 100 μl magnesium chloride (0.06 M) and 245 μl rat liver microsomal preparation. Incubations were carried out with stirring in a water bath at 37 °C. After 30 or 60 min, the reaction was stopped by cooling to 0 °C. TD and its hydroxylated metabolites were extracted into 3 ml of ethyl acetate by shaking in a reciprocal shaker for 10 min. After centrifugation at $2500 \times g$ the organic layer was evaporated under a stream of nitrogen. The residue was dissolved in 30 μl ammonium acetate stock solution and analyzed.

3. Results and discussion

Cyclodextrins, consisting of 6, 7 or 8 D-(+)-glucopyranose units, play a predominant role in chiral CE separations [13–15]. The hydrophilic outside allows the dissolution in the background electrolyte, whereas the lipophilic cavity is responsible for host–guest type interactions leading to the formation of inclusion complexes. The different effective mobilities of the enantiomers are due to the differences in the binding (affinity) constants and this makes the separation of structurally related compounds feasible. Mostly, these chiral selectors are used in a single CD system, but as previous studies illustrate, the use of a mixture of CDs [16–18] may be a valuable extension, especially for the simultaneous enantiomeric separation of diastereomeric compounds. As TD and its hydroxylated metabolites are neutral analytes, they can only be resolved in the presence of charged CD derivatives. Initially, the enantioselective separations were performed in fused-silica capillaries using a single CD system. The best results were obtained using carboxymethyl-β-CD as chiral carrier. In this reversed-polarity mode, the selector also transports the analytes to the detector. Increasing the CD concentration up to 75 mg/ml allowed the improvement of the separation but caused high currents resulting in higher Joule heating. To overcome this problem, a dual chiral selector system, designed in an entirely empirical way, was applied as an alternative technique. Baseline resolution of all enantiomers within an acceptable analysis time could be achieved in a polyacrylamide-coated capillary using 12 mg/ml native β-CD and 12 mg/ml SBE(4)-β-CD in the carrier mode (Fig. 1). The latter selector exhibits a high enantioselectivity for this particular set of analytes. β-CD as uncharged selector does not possess a mobility difference towards the selectand and enhances the chemo- and enantioselectivity of recognition in this separation system. These results confirm that dual CD systems are generally interesting when the two chiral selectors have completely different effects on the analyte mobility.

3.1. Stereoselective in vitro metabolism

As the use of UV detection in capillary electrophoresis has some limitations for high-sensitivity analysis due to the short optical pathlength, attempts were made to extend this detection limit by the use of a higher sample loading. Hydrodynamic injection with 50 mbar for 7 s, dissolution of the incubation residue in 30 μl ammonium acetate buffer at pH 4.5 and pooling of two incubation extracts were found to be the best experimental conditions to increase detection sensitivity without loss in resolution, efficiency and peak symmetry.

Previous studies demonstrated that the in vitro biotransformation of TD by hepatic subcellular fractions resulted in the formation of three chiral hydroxylated metabolites, 5-OH-TD and the diastereomers of 5′-OH-TD, identified by their HPLC retention times, UV- and mass-spectral analyses. Stereoselective effects in metabolism were observed using micellar electrokinetic chromatography (MEKC) as well as achiral HPLC, but the investigation whether formation of the new chiral center on hydroxylation occurs with some degree of enantioselectivity and/or product stereoselectivity requires the use of the chiral CE method. The biotransformation of racemic TD by rat liver microsomes is presented in Fig. 2 (incubation time 30 min). Both enantiomers of all hydroxy metabolites were detected in non-racemic ratios. The results indicate an enantioselective metabolic transformation. The second eluted enantiomer of *trans*-5′-OH-TD (peak 4) is formed predominantly, approximately in a twofold higher

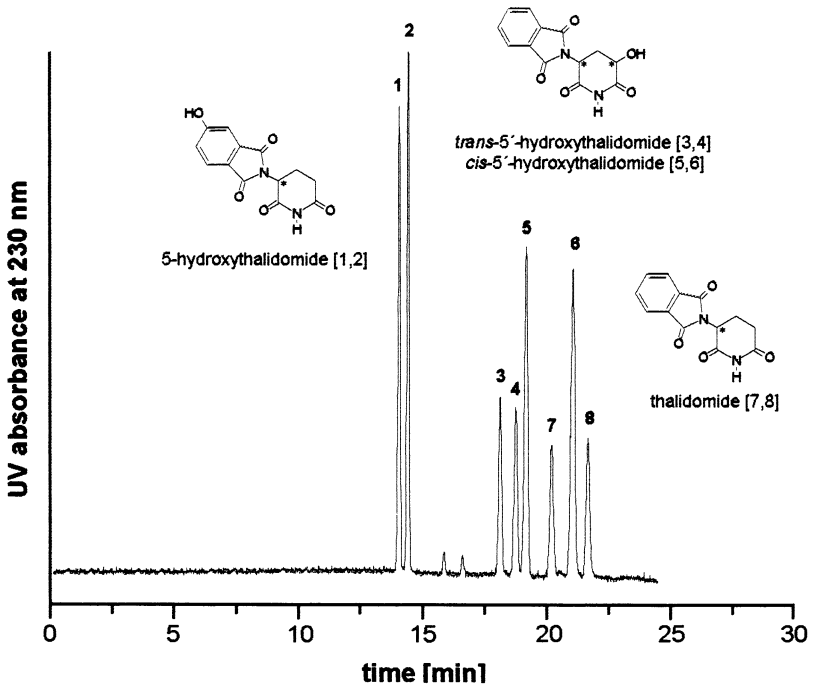

Fig. 1. Simultaneous enantioselective separation of TD and its hydroxylated metabolites (reference compounds) using CE in the presence of 12 mg/ml SBE(4)-β-CD and 12 mg/ml β-CD as chiral selectors. Peak assignments: [1, 2] 5-OH-TD, [3, 4] *trans*-5′-OH-TD, [5, 6] *cis*-5′-OH-TD, [7] S-(−)-TD, [8] R-(+)-TD.

amount than the major enantiomer of the corresponding *cis*-isomer (peak 5). The hydroxylation of the phthalimide moiety of TD was found to be a minor metabolic pathway (peaks 1 and 2).

The unequivocal evidence for the identification of the individual products was made by comparison to the set of standards. Therefore, the incubation residue was additionally spiked with these reference compounds. Metabolites and authentic standards comigrated and consequently no further peaks were observed while the peak heights and areas increased. The formation of the hydroxy metabolites could be abolished when NADPH was omitted from the incubation mixture. This confirms the hypothesis that enzymes of the cytochrome P-450 system of rat liver microsomes, which require NADPH as co-factor for those hydroxylation reactions, are implicated in the metabolism. Furthermore, the electropherogram of a blank incubation extract (without substrate) demonstrates that the products, which were identified as metabolites could not result from the microsomal preparation or from another compound of the incubation mixture.

4. Conclusion

This study provides the first example for the simultaneous separation and enantioseparation of TD and its three hydroxylated metabolites. While baseline resolution for this particular set of analytes could not be

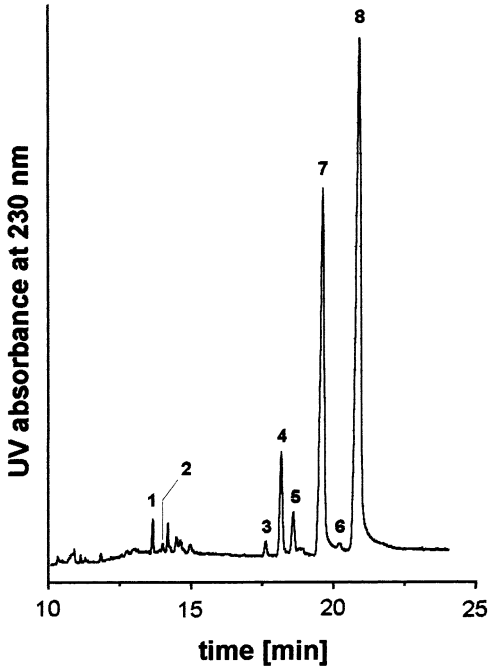

Fig. 2. Simultaneous enantioselective separation of TD and its in vitro metabolites extracted from incubations of racemic TD with rat liver microsomes (incubation time 30 min). Experimental conditions and peak assignments refer to Fig. 1.

achieved either in HPLC, capillary LC or capillary electrochromatography (CEC) on various (e.g. polysaccharide-type) chiral stationary phases, the use of the dual CD system in CE is a chance to separate these compounds in a single run. This chiral CE method presents an opportunity to follow the stereo- and enantioselective effects of the in vitro biotransformation of TD in detail. Our results established the preferential metabolic pathways of R-(+)- and S-(−)-TD and supported the hypothesis of a cytochrome P-450 catalyzed biotransformation. The metabolites found in our incubation extracts with rat liver microsomes were also detected in blood of humans receiving an oral dose of TD as well as in incubations with the human liver fraction S9.

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