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Journal of Chromatography B, 723 (1999) 255–264

JOURNAL OF CHROMATOGRAPHY B

Investigation of the in vitro biotransformation of *R*-(+)-thalidomide by HPLC, nano-HPLC, CEC and HPLC–APCI-MS

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Received 21 July 1998; received in revised form 6 October 1998; accepted 20 October 1998

Abstract

High-performance liquid chromatography (HPLC), nano-HPLC, capillary electrochromatography (CEC) and on-line HPLC–atmospheric pressure chemical ionization mass spectrometry (APCI-MS) techniques were used for the identification and detailed characterization of two new metabolites of the former sedative drug thalidomide (TD). The advantages of nano-HPLC and CEC are higher peak efficiency and a drastic decrease in the analysis time, which, together with lower sample dilution during the analyses, allowed to obtain a detection sensitivity that was comparable to HPLC with common-sized columns. Both, nano-HPLC and CEC could be realized in the commercially available capillary electrophoresis system HP^{3D}. On-line HPLC–APCI-MS coupling is a very useful technique for the rapid identification of metabolites without any need for reference compounds. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Biotransformation; Nano-HPLC; Capillary electrochromatography; Atmospheric pressure chemical ionization mass spectrometry; Thalidomide

1. Introduction

The former sedative drug thalidomide (1,3-dioxo-2[2,6-dioxopiperidin-3-yl]-isoindole; TD) (Fig. 1) was introduced to the market in 1956 under the trade name Contergan® [1]. This drug was withdrawn from clinical practice due to severe toxic effects several decades ago, but use of it is being revived [2]. This is caused by its anti-inflammatory activity for the treatment of leprosy [3] and the recently discovered inhibition of the HIV-1-virus [4]. Furthermore, TD was found to suppress the release of tumor necrosis factor-α (TNF-α) [5,6]. The anti-inflammatory and immunmodulating properties of TD are used in the treatment of graft-versus-host disease following bone marrow transplantation, rheumatoid arthritis, Behçet's syndrome, cachexia in AIDS and several dermatological diseases [2,7].

The main problem that prohibits the clinical application of TD is the severe teratogenic effects of this compound. These toxic effects are mainly expressed in malformations of the limbs and defects of the ears, eyes and internal organs [8]. There are several hypotheses about the mechanisms responsible for the toxic effects. One of the hypotheses involves the metabolic formation of reactive arene oxides,

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PII: S0378-4347(98)00486-1

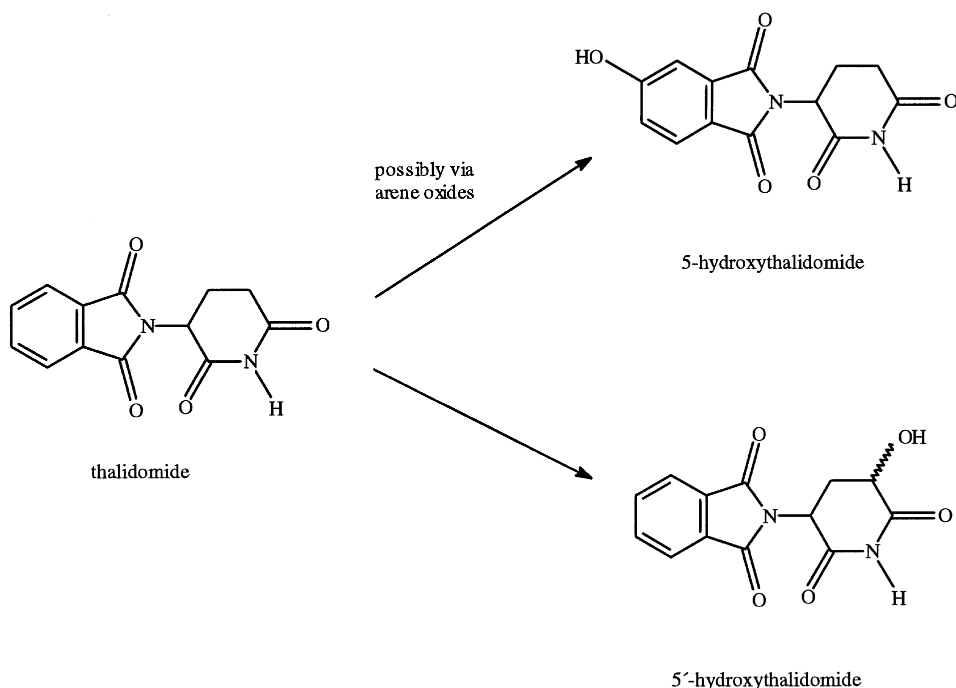

Fig. 1. Metabolic pathways of (*R*, *S*)-thalidomide (TD).

which can be detoxified to less reactive phenols [9]. However, the exact mechanisms of the teratogenic effects are still unknown.

TD possesses a chiral carbon atom in position 1′ of its 2′,6′-dioxopiperidin-3′-yl ring (Fig. 1). Racemisation and metabolic pathways for *S*-(−)-TD have been studied intensively [10–13]. Some indications of the formation of two new diastereomeric metabolites of *R*-(+)-TD, due to hydroxylation at the 5′ position of the 2′,6′-dioxopiperidin-3′-yl ring were observed previously [12,13] (Fig. 1).

The objective of this study was the detailed characterization of the two new diastereomeric metabolites of *R*-(+)-TD. The goal was achieved by the combined use of high-performance liquid chromatography (HPLC), nano-HPLC, capillary electrochromatography (CEC) and on-line HPLC–atmospheric pressure chemical ionization mass spectrometry (APCI/MS) coupling.

2. Experimental

2.1. Chemicals

One of the diastereomers of 5′-hydroxy-TD and the mixture of diasteromeric 5′-acetoxy-TD were gifts from Prof. K. Eger (University of Leipzig, Germany) [14]. *R*-(+)-TD was obtained in our laboratory by preparative scale liquid chromatography on a poly[(*S*)-*N*-(1-cyclohexylethyl)-methacrylamide] stationary phase, as described in ref. [15].

Analytical grade acetic acid, acetonitrile, Tris-(hydroxymethyl)aminomethane (Tris), magnesium chloride, ammonium hydroxide, thiourea and LiChrospher 100 RP-18 (5 μm), for the preparation of packed capillaries, were purchased from Merck (Darmstadt, Germany). NADPH was from Fluka (Fluka Chemie, Buchs, Switzerland) and ethyl acetate was from J.T. Baker (Deventer, The Netherlands).

2.2. Apparatus

Reversed-phase HPLC

The HPLC system consisted of a Knauer Pump 64 (Knauer, Berlin, Germany), a Rheodyne sample injector with a 20-μl loop (Rheodyne, Cotati, CA, USA), a variable-wavelength UV detector L-4000

and a D-2500 Chromato-integrator from Merck-Hitachi (Merck). The analytes were separated on a 125×4 mm RP-18 stationary phase (LiChrospher 100 RP-18, 5 μm; Merck), with a 4×4 mm pre-column. The mobile phase was 1% acetic acid–acetonitrile (90:10, v/v), the flow-rate was 1.2 ml/min and the detection wavelength was 230 nm.

HPLC–APCI-MS

HPLC–MS coupling was performed on a Waters 2690 Separation Module (Waters, Milford, MA, USA) and a LCQ ion trap mass spectrometer equipped with an APCI-interface (Finnigan, Branford, CT, USA), used in the negative ion mode, for the detection of analytes. Separation conditions were the same as in conventional HPLC.

Nano-HPLC

Nano-HPLC experiments were performed using commercially available capillary electrophoresis equipment, HP^{3D} (Hewlett-Packard, Waldbronn, Germany). The separation capillary was a 75-μm I.D. fused-silica column (Polymicro Technologies, Phoenix, AZ, USA) packed with 5 μm reversed-phase material (LiChrospher 100 RP-18) in our laboratory. The total length of the capillary was 33 cm, 24.5 cm were packed with the stationary phase. A mixture of ammonium acetate (5 m*M*, pH 6) and acetonitrile (40:60, v/v) was used as the mobile phase. The sample was introduced by pressure (8 bar) for 12 s. The pressure gradient used for the separation was 12 bar. The detection was performed on-capillary at 220 nm immediately after the outlet frit.

CEC

CEC experiments were performed using the same instrument and the same capillary as in the nano-HPLC experiments. Both pressure (8 bar for 12 s) or electrokinetic injections (7 kV for 5 s) were used in this case. The same mixture of ammonium acetate–acetonitrile as in the case of nano-HPLC was used as a buffer in CEC. The applied voltage used was 25 kV. In CEC and pressure-assisted CEC, 10 bar of pressure were applied at both capillary sides or only at the inlet side, respectively. All separation characteristics (k', α, N, R_s) were calculated using commonly accepted equations in chromatography.

2.3. Methods

Hydrolysis of reference compounds

The hydrolysis of a mixture of both diastereomers of 5′-acetoxy-TD was performed according to ref. [14]. The compound was heated with *p*-toluenesulfonic acid in anhydrous methanol to give a mixture of both diastereomers of 5′-hydroxy-TD.

In vitro biotransformation with rat liver microsomes

Microsomes were obtained from male Sprague-Dawley rats that had been pretreated with 50 mg/kg body weight phenobarbital for six days. After fasting for 24 h, rat livers were prepared according to a method described in ref. [16]. The incubation mixture, with a total volume of 1 ml, consisted of substrate [*R*-(+)-TD, about 100 μg], 0.1 *M* Tris buffer, pH 7.8 (410 μl), the cofactors NADPH and magnesium chloride (245 and 100 μl, respectively) and rat liver microsomal preparation (245 μl). Incubations were carried out with stirring in a water bath at 37°C. The incubation was stopped after 45 or 60 min by cooling to 0°C. TD and its metabolites were extracted with 3 ml of ethyl acetate. After shaking and centrifugation, the organic layer was evaporated under a stream of nitrogen, dissolved and analyzed.

3. Results and discussion

3.1. HPLC

The chromatogram of the sample extracts obtained after the incubation of *R*-(+)-TD with rat liver microsomes (Fig. 2a) and the same sample additionally spiked with the hydrolysis products of diastereomeric 5′-acetoxy-TD (Fig. 2b) are shown in Fig. 2a and b, respectively. These chromatograms show that the metabolites of *R*-(+)-TD and the reference compounds have the same retention time and, therefore, could most likely be identical. The same structure of the metabolites of *R*-(+)-TD were assumed previously, based on HPLC and off-line mass spectrometric studies [12,13].

The obvious disadvantage of the present HPLC method, using a common size RP-18 column, is the very long analysis time and the high consumption of

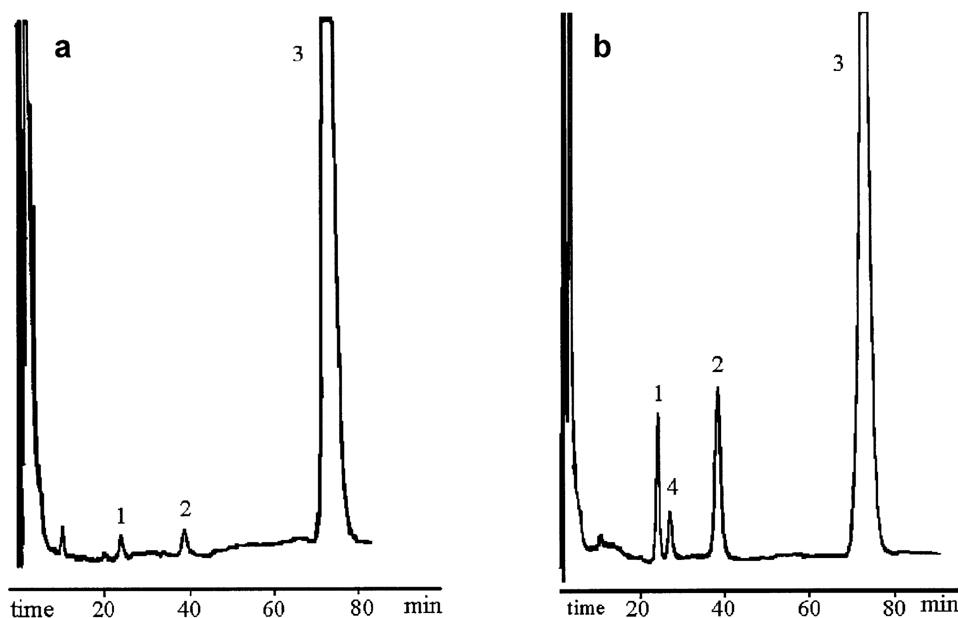

Fig. 2. HPLC separation of *R*-(+)-TD and its metabolites, extracted from incubations (incubation time, 45 min) with rat liver microsomes (a), additionally spiked with reference compound (b). Column, 125×4 mm RP-18 with a 4×4 mm precolumn; mobile phase, 1% acetic acid–acetonitrile (90:10, v/v) at a flow-rate of 1.2 ml/min; UV detection at 230 nm; (1,2) diastereomers of 5′-hydroxy-TD, (3) TD, (4) impurity of hydrolysis.

the hazardous and expensive organic solvent (acetonitrile). To eliminate these problems, attempts were made to miniaturize the HPLC method.

3.2. Nano-HPLC

The nano-HPLC separation of TD and its relatively stable 5′-hydroxy metabolite was performed in a packed fused-silica capillary with 5 m*M* ammonium acetate (pH 6.0)–acetonitrile (40:60, v/v) as the mobile phase. This mobile phase has been selected based on the goal of switching (in further experiments) from nano-HPLC to CEC and to couple both techniques with MS. For this reason, conductivity, relatively high pH (to manage the electroosmotic flow in CEC) and volatility (to avoid contamination of MS) of the buffer were required.

The chromatogram of a mixture of thiourea (which was used as a non-retained compound), 5′-hydroxy-TD and TD using nano-HPLC is shown in Fig. 3. A drastic decrease in the analysis time, an almost fourfold increase in plate number and a marked decrease in the amount of stationary and mobile phase required are the most important advantages of a capillary format compared to a common-size HPLC column. This nano-HPLC experiment was performed in commercially available CE equipment. This equipment uses an external pressure source. The maximal pressure gradient is limited to 12–15 bar. This does not allow to reduce the analysis time further and is therefore a limitation. However, the performance of a nano-HPLC experiment in CE equipment has an important technical advantage. This is the elimination of the need for special (quite sophisticated) sample injection systems as well as micro cells for the detection. At the same time, no connections are required between the injector, the separation column and the detector. This allows for the elimination, in part, of the sources of band-broadening and may contribute to the significantly higher peak efficiency of nano-HPLC compared to HPLC in common-size columns.

In addition, the use of the same experimental setup with the same capillary for the both nano-HPLC and CEC separations allows one to compare these two techniques on a more reliable basis. This means that

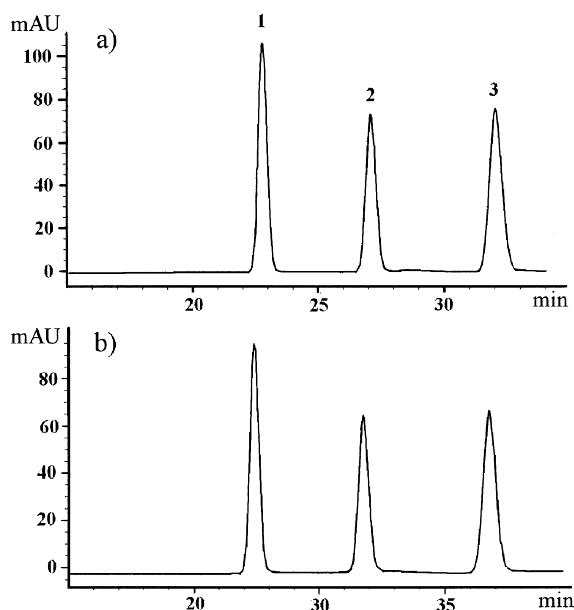

Fig. 3. Nano-HPLC separation of thiourea (dead volume marker) (1), one of the diastereomers of 5′-hydroxy-TD (2) and TD (3). Equipment, HP^{3D} capillary electrophoresis system; capillary, 75 μm (I.D.) fused-silica with a total length of 33 cm packed with 5 μm LiChrospher RP-18 (24.5 cm length); mobile phase, 5 m*M* ammonium acetate–acetonitrile (40:60, v/v); pressure gradient, 12 bar; on-capillary UV-detection, 220 nm; injection, by pressure, 8 bar for 12 s (a) and by voltage, 7 kV for 5 s (b).

the nano-HPLC and CEC separations described in the present work differ only in the migration mechanism of the analytes. All other conditions, e.g. sample injection and detection, dimensions of the capillaries, adsorbent and eluent were exactly the same. This is obviously a very important feature in order to evaluate the potential advantages and disadvantages of the pressure-driven and electrically driven mechanisms in separation science.

The separation of thiourea, one of the diastereomers of 5′-hydroxy-TD and TD is depicted in Fig. 3. It can be seen in this figure that there is no marked difference in peak efficiency regardless of whether the sample is injected by pressure (Fig. 3a) or by electrokinetic injection (Fig. 3b).

3.3. CEC

A CEC separation of the same mixture as that depicted in Fig. 3 is shown in Fig. 4. A further

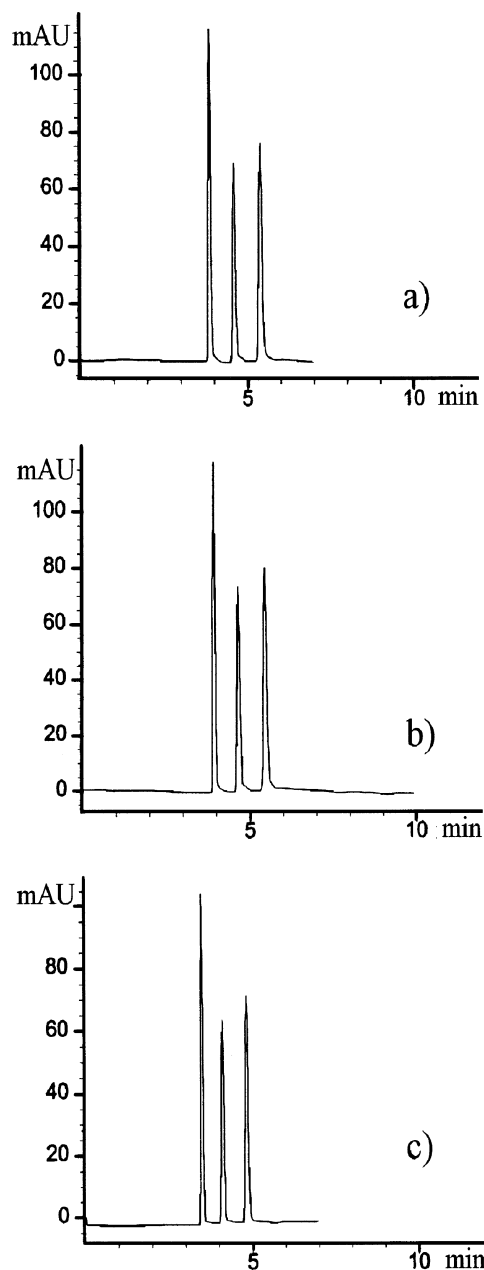

Fig. 4. CEC separation of thiourea (the EOF marker) (1), one of the diastereomers of 5′-hydroxy-TD (2) and TD (3). Injection, by voltage, 7 kV for 5 s. Separation conditions: applied voltage of 25 kV (a), applied pressure of 10 bar at both ends of the capillary; (b) no pressure was applied; (c) an applied pressure of 10 bar only on the inlet side of the capillary. All other conditions as in Fig. 3.

decrease in the analysis time was possible by using the CEC mode. No drastic increase in peak efficiency was achieved by switching from pressure-driven to electrically driven migration mechanisms in this particular case. As mentioned above, the maximal pressure is limited (15 bar) in the HP^{3D} instrument. Therefore, for a more reliable comparison between nano-HPLC and CEC modes, the applied voltage was decreased in the latter technique in order to obtain comparable retention times in both modes (Fig. 5). As shown in this figure, CEC is comparable to nano-HPLC or to a combination of both of these techniques. A further increase in peak efficiency in CEC can be obtained by decreasing the particle size of the packing material. However, this aspect has not been studied in this work.

The data summarized in Table 1 allow us to conclude that CEC is a useful microanalytical technique for the determination of *R*-(+)-TD and its metabolites.

In order to demonstrate the applicability of CEC for a practical problem, the extract from an incubation of *R*-(+)-TD with rat liver microsomes was analyzed (Fig. 6). It was quite surprising to find that, in spite of a shorter optical path length and a markedly lower amount of injected sample, the sensitivity of CEC was comparable to that observed in HPLC with a common-size column. The higher peak efficiencies (sharper peaks) and lower sample dilution in CEC seem to be the most likely reasons for this observation.

The different peak area ratios of diasteromeric 5′-hydroxy-TD (peaks 1 and 2) observed in HPLC (Fig. 2a) and CEC (Fig. 6) chromatograms are caused by the different incubation times of the samples. As observed previously [17], one of the diastereomeric 5′-hydroxy-TD (corresponding to peak 2) is thermodynamically less stable and transforms to another diastereomer (corresponding to peak 1).

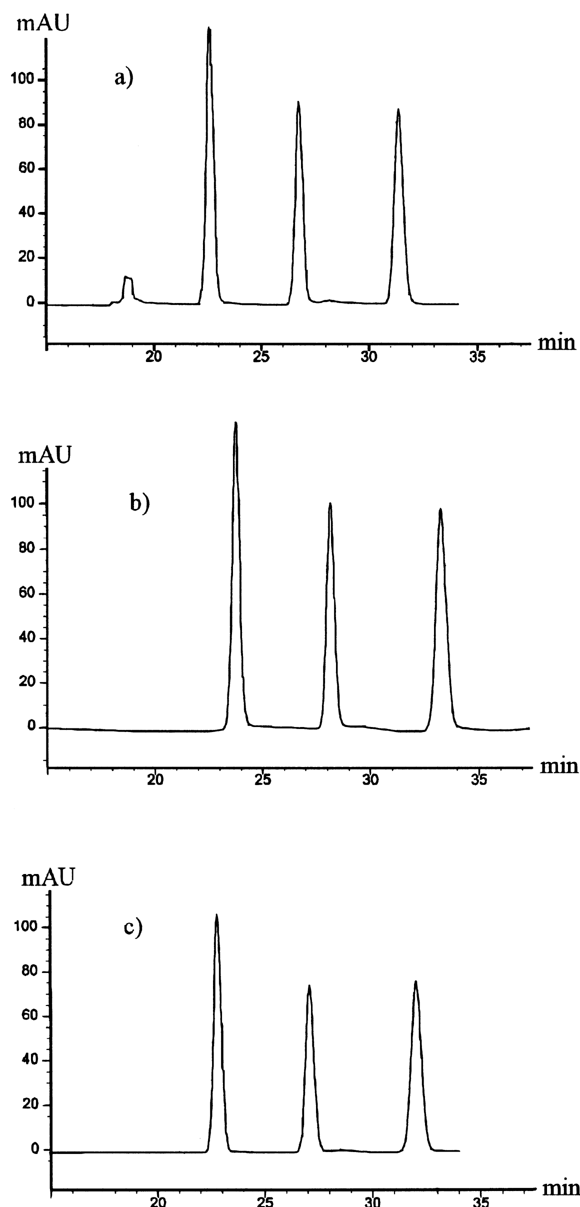

Fig. 5. CEC (a), pressure-assisted CEC (b) and the nano-HPLC (c) separation of a mixture of thiourea (1), 5′-hydroxy-TD (2) and TD (3). Conditions: (a) applied voltage, 5 kV; (b) applied pressure difference, 5 bar, in combination with an applied voltage of 2.5 kV; (c) an applied pressure difference of 10 bar. All other conditions were the same as in Fig. 3.

3.4. HPLC/APCI-MS

One of the basic goals of this study was the on-line identification of the metabolites of *R*-(+)-TD. Initially, the approach was on-line coupling of both capillary techniques with MS using an electrospray ionization interface. However, it was not possible to find conditions in which the ions of TD and its metabolites could be generated easily. In contrast, this was quite easily achieved using an

Table 1
Separation characteristics of a mixture of 5′-hydroxy-TD and TD in CEC

Compound	k'	Separation factor α	Peak efficiency N/m	Resolution R_s
5′-hydroxy-TD	0.18		59 268	
		2.13		4.56
TD	0.39		63 344	

APCI-interface. Several recent papers have described the on-line coupling of APCI with capillary electrophoresis [18]. Unfortunately, the commercial APCI-interface of the LCQ instrument available to us could not be used with the microseparation techniques. Therefore, the identification of TD and its metabolites was performed using on-line coupling of HPLC using a common-size column with APCI-MS (Fig. 7). The concentration of the injected sample that was chosen allowed the detection of R-(+)-TD but hardly the detection of the metabolites, either by UV or MS in the total ion current mode (Fig. 7a). The selected mass-track mode used in the present study allowed us to detect both metabolites, which were identified based on their full scan APCI-MS spectra (Fig. 7b). Currently, studies are in progress to adapt the APCI-interface used in this study for combination with the microseparation methods (CE, nano-HPLC, CEC).

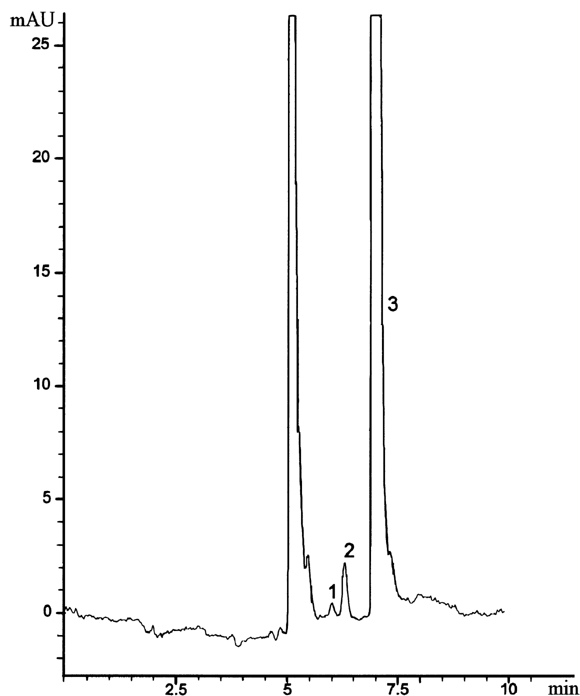

Fig. 6. CEC separation of R-(+)-TD and its metabolites, extracted from incubations (incubation time, 30 min) with rat liver microsomes. Applied voltage, 20 kV. (1,2) Diastereomers of 5′-hydroxy-TD, (3) TD. All other conditions as in Fig. 3.

4. Conclusions

HPLC, nano-HPLC and CEC were successfully used for the separation of thalidomide and its diastereomeric 5′-hydroxy metabolites. Both microanalytical techniques, i.e. nano-HPLC and CEC, can be performed using commercially available capillary electrophoresis equipment and allow the analysis time to be reduced and peak efficiency to be increased. From this viewpoint, CEC offers advantages compared to nano-HPLC. On-line HPLC/APCI-MS coupling confirmed the previously suggested metabolic pathway of R-(+)-TD [12,13]. The major metabolites of R-(+)-TD are the diastereomers of 5′-hydroxy-TD.

Acknowledgements

The authors thank the Deutsche Forschungsgemeinschaft for financial support and Prof. K. Eger for providing the reference compounds. The short-term stay of Dr. Claudia Desiderio at the University of Münster was financially supported by the Consiglio Nazionale delle Ricerche, Rome, Italy.

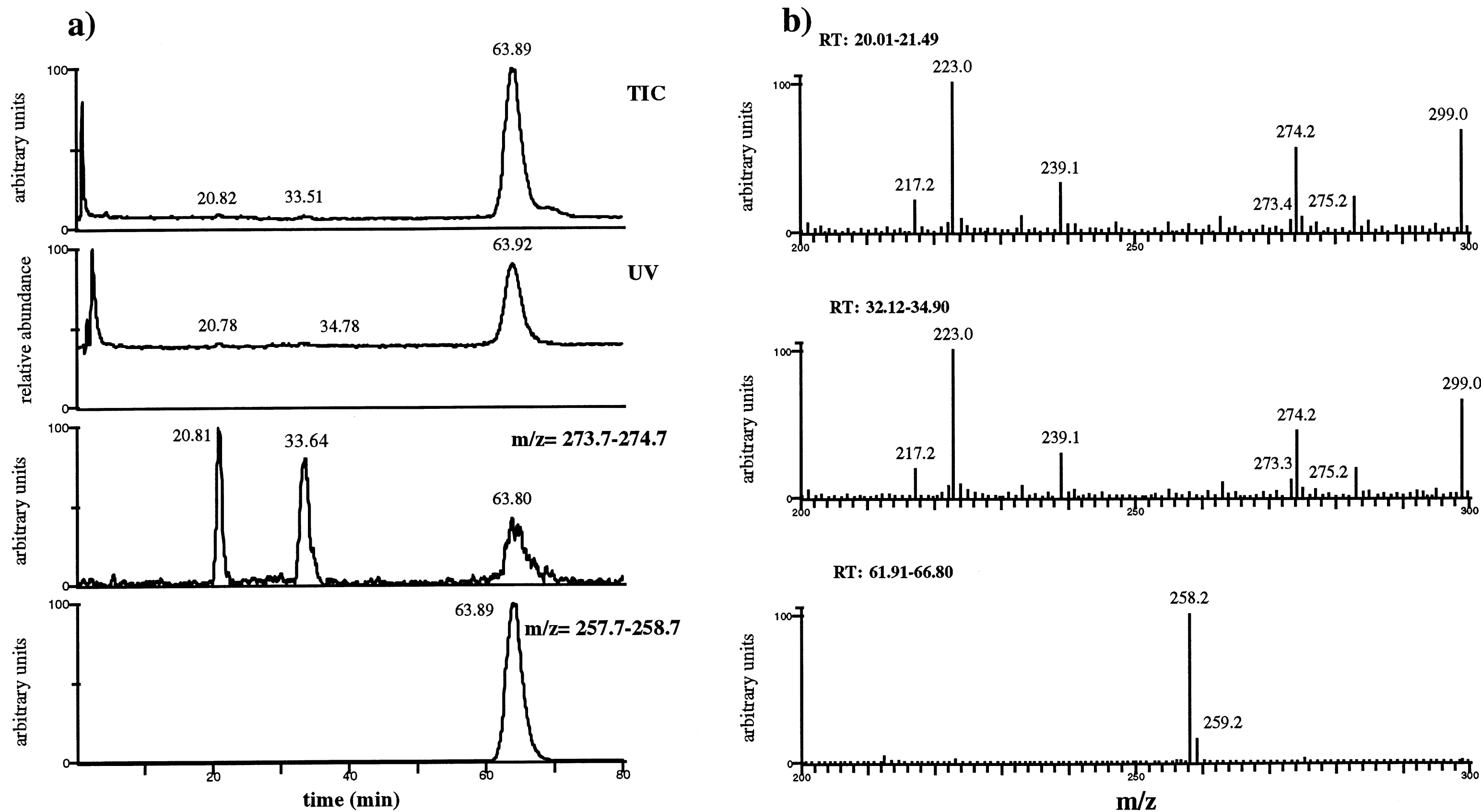

Fig. 7. HPLC/APCI–MS chromatograms of *R*-(+)-TD and its metabolites, extracted from incubations (incubation time, 45 min) with rat liver microsomes [whole mass spectra and selected mass tracks (a) and full scan APCI–MS spectra (b) of selected peaks, Fig. 7a], and additionally spiked with reference compound (Fig. 7b). Conditions for HPLC are as described in Fig. 2.

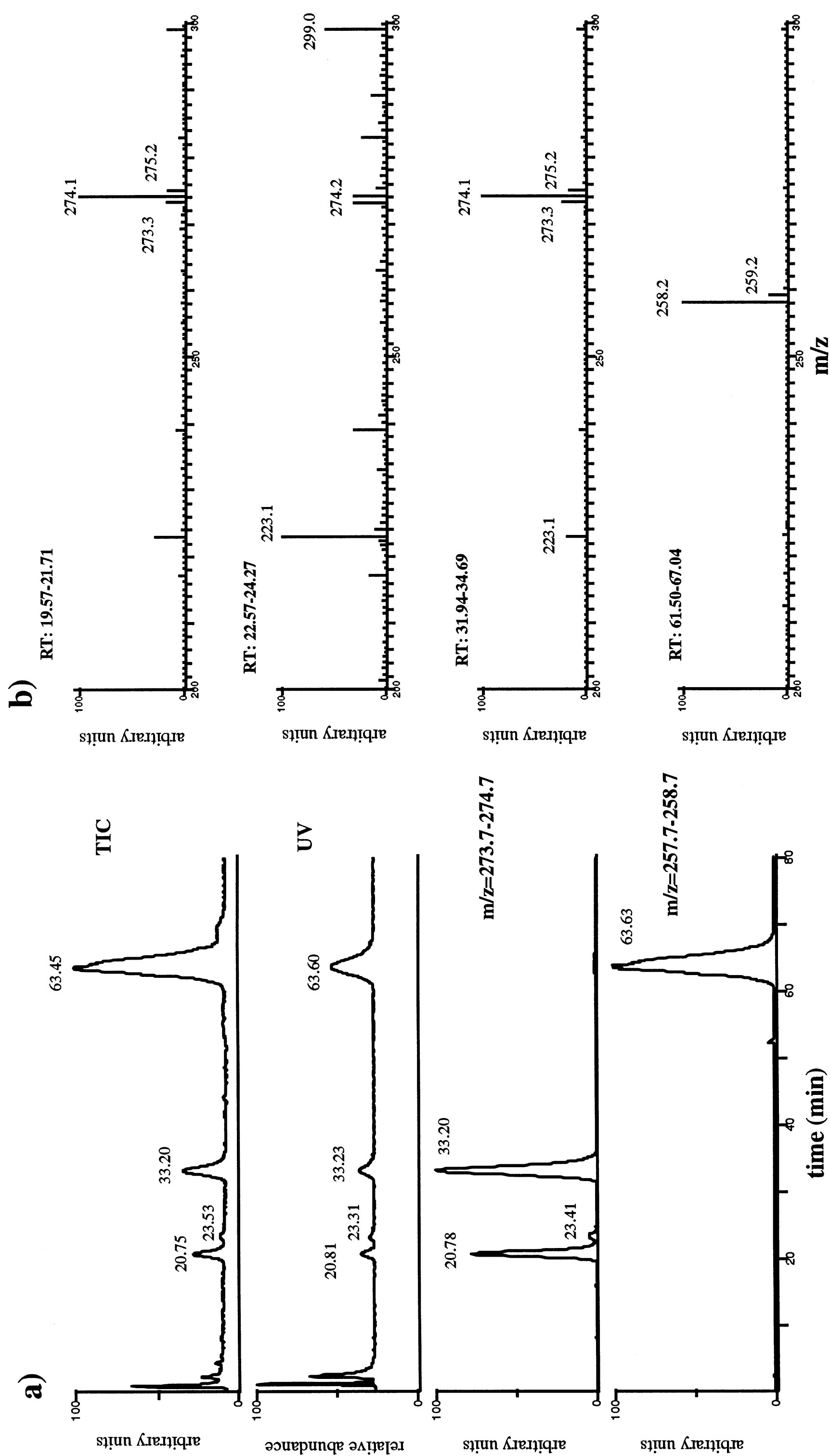

Fig. 7. (*continued*)

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