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Review

Recent advances in analytical determination of thalidomide and its metabolites

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Abstract

Thalidomide, a racemate, is coming into clinical use as immuno-modulating and anti-inflammatory drug. Thalidomide was approved by the FDA in July 1998 for the treatment of erythema nodusum leprosum associated with leprosy. Recently, thalidomide is proving to be a promising drug in the treatment of a number of cancers and inflammatory diseases, such as multiple myeloma, inflammatory bowel disease (Crohn's disease), HIV and cancer associated cachexia. These effects may chiefly be exerted by *S*-thalidomide, but the enantiomers are inter-converted in vivo. Thalidomide is given orally, although parenteral administration would be desirable in some clinical situations.

Thalidomide has been determined in formulations and, principally in biological fluids by a variety of methods such as high-performance liquid chromatography with ultraviolet detection and liquid chromatography coupled with tandem mass spectrometry. The overview includes the most relevant analytical methodologies used in its determination.

Keywords: Thalidomide; Review; Enantiomers; Biological samples

Contents

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1. Introduction

Thalidomide (TD), 1,3-dioxo-2-[2′,6′-dioxopiperidin-3′-yl]-isoindol, is a sedative, hypnotic, and anti-inflammatory medication. TD was developed by German pharmaceutical company Grünenthal. It was sold from 1957 to 1961 in almost 50 countries under at least 40 names, including Distaval, Talimol, Nibrol, Sedimide, Quietoplex, Contergan, Neurosedyn, and Softenon. TD was chiefly sold and prescribed during the late 1950s and early 1960s to pregnant women, as an antiemetic to combat morning sickness and as an aid to help them sleep. Before its release, inadequate tests were performed to assess the drug's safety, with catastrophic results for the children of women who had taken TD during their pregnancies.

From 1956 to 1962, approximately 10,000 children were born with severe malformations, including phocomelia, because their mothers had taken TD during pregnancy. In 1962, in reaction to the tragedy, the United States Congress enacted laws requiring tests for safety during pregnancy before a drug can receive approval for sale in the U.S. Other countries enacted similar legislation, and TD was not prescribed or sold for decades.

There are few topics in biological and pharmaceutical science that have drawn as much interest as the chiral nature of drug molecules. Today, systematic investigation of the biological activity, including pharmacology and toxicology, of individual enantiomers is the rule for all new racemic drug candidates and an increasing number of optically pure drugs has been approved and marketed.

TD has a chiral center in its structure and is synthesized as the racemate. Enantiomers often do not possess the same physiological effects; therefore analytical methods are required to discriminate between the two isomeric biomolecules. In the case of TD, it was later determined that the teratogenic effects were mainly caused by the *S*-enantiomer of thalidomide. As can be seen in posterior sections of this paper, Eriksson and co-workers have contributed significantly to the analysis, distribution and biotransformation of both enantiomers of TD.

Researchers, however, continued to work with the drug. Soon after its banishment, a doctor discovered anti-inflammatory effects of TD and began to look for uses of the medication despite its teratogenic effects. They found that patients with erythema nodosum leprosum, a painful skin condition associated with leprosy, experienced relief of their pain by taking TD. There are studies underway to determine the drug's effects on arachnoiditis, Crohn's disease, Behçet's syndrome, and conditions associated with HIV infections including aphthous ulcers and wasting syndrome, graft-versus-host disease, multiple myeloma and several types of cancers. However, physicians and patients alike must go through a special process to prescribe and receive TD to ensure no more children are born with birth defects traceable to the medication.

The renewed interest that thalidomide (α-phthalimidoglutarimide) presently attracts as an anti-inflammatory agent and as the drug of choice for the treatment of the lepra reaction, as well as of various other diseases of the skin and the mucous membranes, requires the development of rapid and sensitive quantitative assays for both the parent compound and its metabolites (Fig. 1) in biological material, such as blood serum or tissue samples.

Earlier investigations on the teratogenicity of TD demonstrated that after application in rats and mice only *S*-(−)-thalidomide was teratogenic. However, it is known that TD is sensitive to hydrolytic decomposition and that it undergoes racemization. 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, a rate which dramatically increases with elevations of either pH or temperature. Previous studies showed that TD re-isolated from plasma 2 h after intravenous application of *S*-(−)-thalidomide is completely racemixed. It has also been suggested that TD enantiomers can interconvert in the body, indicating that the exclusive use of the *R*-enantiomer is not enough to prevent these undesirable side effects. The aqueous solubility of racemic TD was reported to be very low, around 50 μg mL^{-1}.

2. High-performance liquid chromatography (HPLC)

2.1. Pharmaceutical formulations

A method, described by Reepmeyer and Cox [1], uses a Nova-Pak octadecylsilane bonded-phase column (150 mm × 3.9 mm, 4 μm particle size), a mobile phase of acetonitrile–water (15:85), a flow rate of 1 mL min^{-1}, detection at 237 nm, and phenacetin as internal standard. Phosphoric acid was used in preparation of sample solutions to inhibit TD hydrolysis. The method has been used to analyze individual tablets and capsules for determination of content uniformity.

2.2. Biological samples

Czejka and Koch report the separation and quantification of TD and its major metabolites after extraction from biological material (human serum) using isocratic and reversed-phase chromatography and tetramethylammonium bromide as an ion-pair forming reagent [2], but the dynamic range of this assay was 1–20 μg mL^{-1}, which is well above the plasma concentrations of 0.2–1.4 μg mL^{-1} attained during the first 24 h after a single therapeutic dose [3]. The HPLC assay used in the latter study had a sufficiently low linear range of 0.1–10 μg mL^{-1}. For a third HPLC method [4], about effect of TD treatment for chronic graft-versus-host disease, linear range, accuracy and precision were not reported. In the same way, other proposed method uses 0.5 mL plasma, is linear to 10 mg L^{-1} and had a detection limit of 0.2 μg mL^{-1} [5]. TD in plasma specimens was unstable at physiological pH but could be stabilized for several weeks by simple acidification. The authors describe a protocol for monitoring

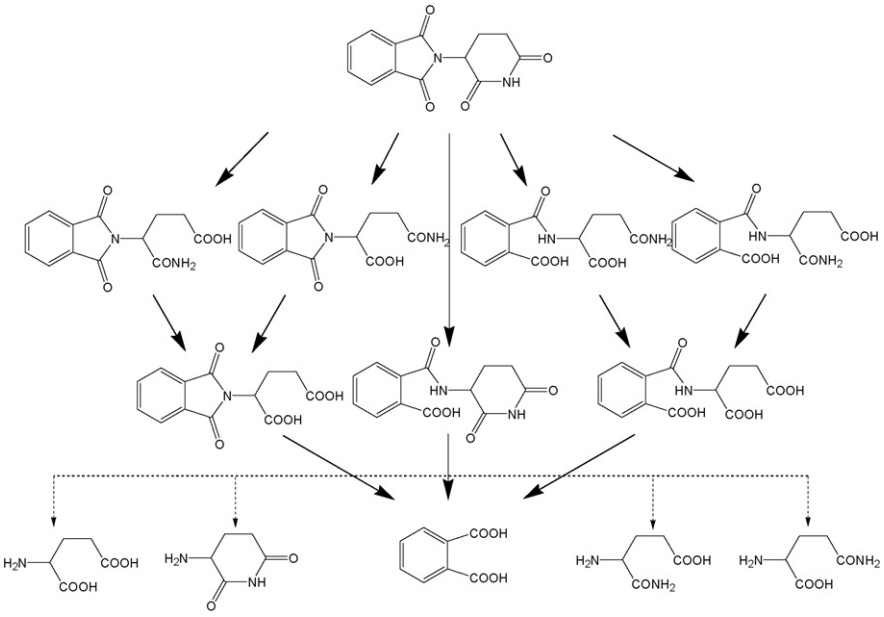

Fig. 1. Scheme of the hydrolysis of thalidomide.

patients treated with TD which permits convenient transportation and storage of specimens and report, provisionally, that plasma concentrations in the range 1–7 $\mu g\,mL^{-1}$ are therapeutically effective in chronic graft-versus-host disease without adverse side effects. The authors collected blood in heparin tubes and centrifuged within 15 min. Aliquots of plasma were then transferred to tubes containing twice the volume of 1 M HCl. With this treatment, TD was stable for several weeks even at room temperature.

Delon et al. [6] developed a procedure for the analysis of TD in plasma that involved a single liquid–solid extraction on Extra-Sep-C_8 column in the presence of an internal standard (ciprofloxacin). Analysis was performed by isocratic elution with a mobile phase consisted of 0.01 M aqueous potassium dihydrogen phosphate containing 21% acetonitrile and 4.5 mM heptane sulfonic acid, adjusted to pH 2.3, with UV detection at 295 nm. The limit of sensitivity of the assay was 0.06 $\mu g\,L^{-1}$. The method was applied to a pharmacokinetic study in patients with erythema nodosum leprosum.

Also, Hewlett-Packard 1090 Series II Liquid Chromatograph equipped with a photodiode–array detector was used for the chromatographic analysis [7]. A Waters Nova-Pak C_{18} (3.9 mm × 300 mm) column was used. TD and phenacetin (internal standard) were detected at UV wavelengths of 220 and 248 nm, respectively, with a run time of 16 min. A gradient mobile phase of water, acetonitrile, and a 0.5 M NaH_2PO_4 buffer (pH 3.0) was run at a how rate of 1 mL min^{-1}. TD was isolated from serum by solid phase extraction. Ten percentage of H_2SO_4 (7.5 μL) was added to the serum to halt degradation. Later, a HPLC method was developed to determine racemic TD in plasma on an avidin protein column using solid phase extraction [8]. The enantiomers were separated isocratically using a mobile phase of 2:98 2-propanol:phosphate buffer (0.1 M, pH 4) at a flow rate of 0.6 mL min^{-1} at ambient temperature with detection at 300 nm; the chromatographic run time was less than 13 min; the limits of detection were 100 and 50 ng mL^{-1}, respectively, for each enantiomer.

TD was determined in plasma and blood by reversed-phase with ultraviolet detection and a study about the prevention of hydrolytic degradation of thalidomide in samples and stock solutions are realized [9]. The coefficient of variation of the assay was 1–2% over the 0.25–4.0 $\mu g\,mL^{-1}$ concentration range. Hydrolysis of thalidomide during storage and work-up of the samples was avoided by the addition of an equal volume of citrate buffer, pH 1.5. In this way, Lyon et al. [10] realised other work with the aim to assess and adapt this method for the application of drug monitoring during a clinical trial; they proposed immersing blood samples in an ice-slush filled cup to take to a clinical laboratory for centrifugation. The plasma aliquots are then transported and stored at −25 °C until analysis. With this procedure, the fraction of TD remaining after 30 days was calculated to 0.90. Huupponen and Pyykko [11] recommended that blood samples must be handled refrigerated and the plasma separated promptly, but they did not address the issue of degradation of TD later during storage of the samples.

Eriksson and Björkman [12] describe a sample handling procedure in detail: (a) add 2.00 mL of 25 mM citrate buffer (pH 1.5) to 10 mL glass-stoppered extraction tubes; (b) collect blood in anticoagulated (with heparin) evacuated tubes; (c) without delay, transfer 2.00 mL of blood to each extraction tube, and mix immediately; (d) as soon as possible, freeze and store at −25 °C; (e) analyze all samples within 75 days.

Since the main site of biotransformation of the enantiomers of TD is apparently circulating plasma or blood, an understanding of the pharmacokinetics of TD in vivo requires thorough knowledge of its reactions in vitro, in the presence of blood and plasma components. In this sense, Eriksson et al. [13] realized a study with the aim to characterize the distribution in blood, i.e., the plasma protein binding, the blood/plasma concentration ratio, and the erythrocyte/plasma distribution of the enantiomers

of TD, as well as the influence of pH and plasma proteins on their simultaneous inversion and hydrolysis. In order to obtain possible hints as to the mechanism by which human serum albumin (HSA) catalyses the inversion of the enantiomers, this reaction was also studied with addition of some putative inhibitors. A stereo-specific HPLC method for determination of concentrations of the separate enantiomers has been described previously [14], as well as a non-stereo-specific method for determination of total TD concentrations [9]. For both methods, UV detection at 220 nm was used. In brief, the total concentration of TD was determined by HPLC using an RP-18 column and a mobile phase of acetonitrile:water (35:65). Latter, the same research group realised a study with the aim to prepare solutions of the enantiomers of TD for intravenous administration and to investigate their pharmacokinetics and sedative effects following infusion in man. Solubility and stability of the enantiomers in 5% glucose solution was also investigated. Blood concentrations of the enantiomers were determined by stereo-specific HPLC; this is the first time that TD has been administrated intravenously [15].

TD enantiomers have been separated by HPLC with chiral stationary phases consisting of a chiral polyamide [16], a benzoyl derivative of cellulose (Chiral Tribencel) [14] and a methylbenzoyl derivative of cellulose (Chiralcel OJ) [17]. In this last case, a simple isocratic and direct resolution of racemic TD was accomplished without any derivatization. Solvent system was hexane and ethanol (50:50) with the flow rate 1 mL min^{-1} at 23 °C. The capacity factor (k') for first eluted enantiomer *R*-(+)-thalidomide was 9.67 and separation factor (α) obtained was 1.54. The maximum stereochemical resolution factor (*R*) obtained was 15.05. The method was applied for optical purity determination of the drug in bulk and formulation dosage forms.

Racemisation studies of TD after incubation of the enantiomers in different aqueous media were made by Knoche and Blaschke [18]. The enantiomeric ratio after extraction from these media was determined by chiral HPLC. Thalidomide enantiomers were separated on a 250 mm × 4 mm poly[(*S*)-*N*-(l-cyclohexylethyl) methacrylamide] stationary phase which was immobilized on modified silica, equipped with a 30 mm × 4 mm LiChrospher 60 guard column. The mobile phase was *n*-hexane–dioxane (7:3), the flow rate 1.0 mL min^{-1} and the detection wavelength 230 nm.

The purposes of work realised by Eriksson et al. [14] were: (a) to develop a HPLC assay for the enantiomers of TD in blood; (b) to study their inversion and degradation in human blood; (c) to study the pharmacokinetics of (+)-(*R*)- and (−)-(*S*)-TD after oral administration of the separate enantiomers or of the racemate to healthy male volunteers.

Also, cyclodextrins have been used for the attainment of enantiomeric separations in HPLC, either as bonded to the stationary phase or used as an additive in the mobile phase. TD is poorly soluble in water, but has been solubilised and stabilized in aqueous solutions by the addition of hydroxypropyl-β-cyclodextrin. Enantiomers of TD are resolved by HPLC on a C_{18} column using β-cyclodextrin as a mobile phase additive. This approach provides a means for separating *R*- and *S*-TD without requiring a special chiral column [19].

Previously, the chiral α_1-acid glycoprotein (AGP) column has been used to separate other drug enantiomers with short retention times; based in this fact, Álvarez et al. [20] develop a rapid and enantioselective HPLC assay for TD samples employing an immobilized AGP column; in this case, the best mobile phase was pH 7.0, 30 mM ammonium acetate containing 0.3% tetrahydrofuran.

Such as we describe previously, TD degrades in aqueous media, depending on pH and temperature. Thus, the proper handling of blood or plasma samples is crucial. Yang et al. [21] develop a HPLC method for determination of TD in rat plasma and applied it to the pharmacokinetic studies in combination with irinotecan (CPT-11). TD may modulate the metabolism and transport of CPT-11 and thus alter its pharmacokinetics in vivo. Chromatography was accomplished with a reversed-phase Hypersil C_{18} column. Mobile phase consisted of acetonitrile:10 mM ammonium acetate buffer (28:72; pH 5.5), at a flow rate of 0.8 mL min^{-1}. TD was monitored at 220 nm and it gave a linear response over 0.02–50 μM range.

In an attempt to characterize the transport of TD by the human colon cancer cell line (Caco-2 cells), which have been widly used to investigate drug permeability and transport, Zhou et al. [22] developed simple HPLC methods for the determination of TD in the transport buffer for the human colonic cell line (Caco-2) cell monolayers. An aliquot of 50 μL of the mixture was injected onto a Spherex C_{18} column (150 mm × 4.6 mm; 5 μm) at a flow rate of 0.5 mL min^{-1} of mobile phase consisting of acetonitrile 10 mM ammonium acetate buffer (24:76, pH 5.5), and TD was detected by UV detector at a wavelength of 220 nm. Calibration curves for TD were constructed at the concentration range of 0.025–1.0 and 1.0–50 μM in transport buffer. The validated methods were used to determine the transport of thalidomide by Caco-2 monolayers. The transport across the monolayers from the apical (A) to basolateral (B) side was similar to that from B to A side.

In order to avoid the hydrolytic degradation of TD during sample preparation, trichloroacetic acid was used to protein precipitation with 78–81% recovery [23]. The aliquot was chromatographed on an octadecyl column, using an eluent composed of 250 mL 0.01 M potassium dihydrogenphosphate, adjusted to a pH of 3.0 with a 43% phosphoric acid solution, mixed with 750 mL methanol, with phenacetin as an internal standard. UV detection was used at 220 nm. Hydrolitic degradation was prevented during analysis by acidification of samples with the precipitation reagent. Thalidomide and phenacetin were found to have retention times of 7.9 and 15.0 min, respectively. This procedure and others HPLC coupled with UV methods cited previously [2,9,22,23] measured the total form (protein-bound plus unbound) drug concentrations. In contrast, obtaining protein-free drug analyte could be carried out by either ultrafiltration or the microdialysis method, but ultrafiltration has some limitations which make it difficult to apply in some in vivo studies of neurotransmitters and pharmacokinetics of unbound drugs in the brain due to its larger membrane loop. Microdialysis provides numerous advantages, such as offering clean samples for direct analysis and allowing continuous monitoring of analytes for much higher spatial and temporal resolution, and better tissue pharmacoki-

netic studies. In this way, a microdialysis system was utilised with multiple probes to construct a free-form drug sampling coupled with HPLC–UV detection to concurrently monitor unbound TD in rat blood, brain and bile for pharmacokinetic study [24]. Microdialysis probes were concurrently inserted into the jugular vein toward the right atrium, the brain striatum and the bile duct of the anesthetized Sprague–Dawley rats for biological fluid sampling after the administration of TD (5 mg kg^{-1}) through the femoral vein. TD and dialysates were separated using a Zorbax ODS C_{18} column (150 mm × 4.6 mm; particle size 5 μm) maintained at ambient temperature. The mobile phase was comprised of acetonitrile–methanol–0.1 mM 1-octanesulfonic acid (32:3:65; pH 5.3), and the flow arte of the mobile phase was 1 mL min^{-1}. The buffer was filtered through a Millipore 0.45 μm filter and degassed prior to use. The UV detector was set at 220 nm.

As can be see, many methods have been developed to quantify TD and its metabolites from a variety of biological matrices. Most of these methods are achiral, being based on HPLC using reverse phase separation with UV, MS or tandem MS detection. However, increasing recognition of the potential importance of stereoselectivity in TD pharmacology has, more recently, led many investigators to separate its enantiomers, in some cases also with separation of various metabolites. Such approaches are commonly based on chiral stationary phase (CSP)–HPLC, capillary electrophoresis (CE) or electrochromatography (CEC) technology. Vancomycin, a macrocyclic antibiotic, has been utilized as a CSP for separating a number of racemic drugs, including TD; however, there has yet been no reported use of a vancomycin–CSP for quantitative analysis of TD concentrations in biological samples. For this reason, a quantitative chiral HPLC method was required to determine its enantiomers in serum, tumours and tissues of animals treated with TD and combinations of TD and chemotherapy, as well as in serum of patients undergoing TD treatment. As no published methods were available for the routine and high throughput determination of TD enantiomers in relevant biological samples, Murphy-Poulton et al. [25] developed and validated this relatively simple method of liquid/liquid extraction of TD, with vancomycin HPLC–CSP for enantiomer separation, and UV detector determination. TD in relevant serum and tissue homogenate samples was stabilized by buffering with an equal volume of citrate-phosphate buffer (pH 2, 0.2 M), and stored at −80 °C pending assay. The TD enantiomers, extracted from the samples with diethyl ether, were well separated on a chiral HPLC column of vancomycin stationary phase and a mobile phase of 14% acetonitrile in 20 mM ammonium formate adjusted to pH 5.4; their concentrations were determined with phenacetin as internal standard at 220 nm detection. The estimated limit of quantification for both enantiomers was 0.05 μg mL^{-1} with 0.2–0.6 mL serum samples. TD in rat and human serum, acidified and stored as described above, was found to be chemically and chirally stable over 1 year. The method has been successfully applied to serum samples from human patients undergoing TD treatment for mesothelioma, and to serum, blood and tissue samples from a laboratory rodent model using transplanted 9 L gliosarcoma.

In other study, several cyclic imidic compounds, including TD, are enantiomerically resolved via HPLC on a macrocyclic antibiotic covalently bonded to a silica gel support. The Chirobiotic V chiral stationary phase column contains the antibiotic vancomycin as the chiral selector. The results of the analysis show that the substituents at the chiral carbon position of the racemic drugs affect chiral resolution [26].

2.3. LC–MS

Svensson et al. [27] use a chromatographic system similar to that described in a previous article. A pump operated in constant pressure mode was connected to an injector equipped with a 2 mL mobile phase loop followed by a sample injector with an internal loop volume of 0.2 μL. The columns were connected to a UV detector using a 50 μm i.d. fused silica tube. LC–MS and LC–MS/MS were performed with a Finnigan TSQ 700 triple quadrupole mass spectrometer equipped with an electrospray ion source. An in-house-made spray emitter was used with a flow of 3–5 μL min^{-1} and the spray voltage was 4.5 kV in all experiments. The heated capillary was set at a temperature of 220 °C. Acetonitrile mixed with ammonium acetate buffer 5 mM or acetic acid 0.1 M was used as mobile phases.

Teo et al. [28] describe highly sensitive, rapid, selective and high throughput LC–MS/MS assays of TD in human plasma and semen with calibration range of 2–250 ng mL^{-1}. The matrices were first stabilized with 0.025 M Sorensen's citrate buffer at pH 1.5 to prevent spontaneous hydrolysis. Buffered TD was stable when stored at room temperature for 24 h and for up to three freeze–thaw cycles. Samples were extracted using SPE cartridges. Extracts were then injected into the LC–MS/MS equipped with a reversed-phase column and APCI interface in the negative ion mode. The method successfully determined concentrations of TD from a clinical study to levels as low as 7 ng mL^{-1} plasma and 8 ng g^{-1} semen.

2.4. Hydroxylated metabolites

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.

Notably, the enantiomers of TD show a significant enantioselectivity in pharmacodynamics. The immunomodulatory effects may chiefly be exerted by (*S*)-TD, whereas sedation was related to the blood concentration of the (*R*) enantiomer. About four decades after Lenz and McBride independently associated the use of TD in early pregnancy with the occurrence of phocomelia, the exact mechanism of teratogenesis remains unknown.

Several different hypotheses were discussed, suggesting, for example, the bioactivation of TD by embryonic prostaglandin H synthase, which causes oxidative damage to DNA. The latest results proposed that the drug affects the pathway of insulin-like growth factor 1 and fibroblast growth factor 2 stimulation of α-v and β3 integrin subunit genes during development.

Another tenable explanation of the teratogenic effects involves the metabolic formation of reactive arene oxide intermediates, which are detoxified to (less reactive) phenols.

The stereoselective metabolism of TD and the metabolism of its analogue EM 12 were studied in vitro with liver homogenates [29]. This study is focused on hydroxylated nonhydrolyzed metabolites of TD. A HPLC method was developed to determine these metabolites directly. The investigations showed a highly stereoselective biotransformation of TD. 5-Hydroxy thalidomide was preferentially formed by (−)-(*S*)-TD, whereas (+)-(*R*)-TD was metabolized to two hitherto unknown compounds (Met A and B). Mass spectrometry of these metabolites Met A and B indicated that oxidation or hydroxylation took place in the glutarimide moiety. Biotransformation studies with the more stable TD analogue EM 12 revealed four new metabolites (Met C–F) whose quantities differed in the selected liver homogenate.

The objective of a study realized by Meyring et al. [30] was the detailed characterization of the two new diastereomeric metabolites of *R*-(+)-TD. The goal was achieved by the combined use of HPLC, nano-HPLC, capillary electrochromatography (CEC) and on-line HPLC–atmospheric pressure chemical ionization mass spectrometry (APCI/MS) coupling. 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 obtaining detection sensitivity that, was comparable to HPLC with common-sized columns. Also, Meyring et al. [31] carried out the separation of TD and its hydroxylated metabolites including their simultaneous enantioseparation using three different polysaccharide-type chiral stationary phases (CSPs) in combination with polar organic mobile phases. Three different techniques, HPLC in common-size columns, capillary LC and nonaqueous capillary electrochromatography were compared in terms of separation. As this study illustrates, polar organic mobile phases represent a valuable extension for less polar and polar aqueous–organic mobile phases in combination with polysaccharide CSPs. Chiralpak AD consisting of 25% of amylose-tris(3,5-dimethylphenylcarbamate) coated on wide-pore aminopropylsilanized silica gel exhibited higher resolving ability compared to the similar cellulose derivative (Chiralcel OD) as well as to cellulose-tris(4-methylbenzoate) (Chiralcel OJ) CSPs for this particular set of chiral analytes.

The application of TD in an in vitro lymphocyte assay system provided the evidence that the drug itself was not toxic, whereas toxicity required both, a source of hepatic enzymes and a NADPH-generating system. Although TD was found to be a poor substrate for cytochrome P-450, Meyring et al. [32] focused a work on the enzyme-catalyzed in vitro biotransformation by rat liver microsomes due to the observations mentioned above. The metabolites previously detected in these incubation extracts [33,34] were also found in plasma samples from male volunteers who had received TD orally [35].

Former studies using capillary electrophoresis with cyclodextrins as chiral selectors allowed the enantioselective separation of TD and three hydroxylated derivatives [34]. These investigations demonstrated the stereoselective in vitro biotransformation: the (*R*) enantiomer is preferentially metabolized in the 2′,6′-dioxopiperidine-3′-yl moiety, whereas (*S*)-TD is mainly transformed by hydroxylation in the phthalimide ring.

This research group describes the detailed stereochemical characterization of the hydroxylated derivatives of TD by means of experimental circular dichroism (CD) spectroscopy in combination with quantum chemical CD calculations [32]. Incubation extracts of the individual enantiomers were analyzed by HPLC on an achiral stationary phase combined with CD detection. The CD data of the almost enantiopure eluates of the metabolites were compared with the CD spectra quantum chemically calculated for the respective structures. (3′*R*,5′*R*)-*trans*-5′-hydroxythalidomide is the main metabolite of (*R*)-TD, which epimerizes spontaneously to give the more stable (3′*S*,5′*R*)-*cis* isomer. On the contrary, (*S*)-TD is preferentially metabolized by hydroxylation in the phthalimide moiety, resulting in the formation of (*S*)-5-hydroxythalidomide.

2.5. Determination of the thalidomide analogues 2-(2,6-dioxopiperidine-3-yl) phthalimidine (EM 12), 2-(2,6-dioxopiperidine-4-yl) phthalimidine (EM 16) and their metabolites

Although there are structural similarities between TD, EM 12 and EM 16 there are great differences in their teratogenic potential and in the number of metabolites they produce (at least 12 from TD, 3 from EM 12; and just 2 from EM 16). Following an optional extraction, samples were analysed by reversed-phase HPLC with ion depression. The recovery of the extraction procedures was 65–80%. The method has been applied to pharmacokinetic studies in small laboratory animals and in vitro model experiments [36].

2.6. Determination of CC-5013

Such as we comment previously, TD has been shown to be effective in a variety of tumour types, and it was found that TD inhibits tumour growth at least in part by anti-angiogenic mechanisms. More recently, analogues of TD have been developed that are more potent inhibitors of angiogenesis. CC-5013 [α-(3-aminophthalimido) glutarimide; lenalidomide], an immunomodulatory TD analogue, has demonstrated higher potency than TD in the human umbilical vein endothelial cells (HUVEC) proliferation and tube formation assays. These assays also indicated a dose-dependent decrease in HUVEC proliferation and tube formation with increasing concentration of CC-5013. Tohnya et al. [37] describe a LC–MS assay for determination of this compound in human plasma. Sample pre-treatment involved liquid–liquid extraction with acetonitrile:1-chlorobutane (4:1) solution containing the internal standard, umbelliferone. Separation of the compounds of interest was achieved on a column packed with Waters C_{18} Nova-Pak material (4 μm, particle size; 300 mm × 3.9 mm) using acetonitrile, deionised water, and glacial acetic acid (20:80:0.1; pH 3.5) delivered at an isocratic flow rate of 1.0 mL min^{-1}. Simultane-

ous MS detection was performed at *m/z* 260.3 (CC-5013) and 163.1 (umbelliferone).

2.7. Determination of glutamine

The TD molecule, a synthetic derivative of glutamine, can undergo hydrolysis at physiologic pH to form glutamine as a degradant. Additionally, L-glutamine is one of the starting materials in the synthesis of TD drug substances. Therefore, glutamine is monitored in both TD drug substance and pharmaceutical dosage forms. The method currently used to determine glutamine in TD drug substance and products is thin-layer chromatography (TLC) with ninhydrin spray visualization [38]. The TLC method was not automated and only semi-quantitative due to the manual introduction of sample and visual detection. Therefore, it was desired to explore alternative, quantitative chromatographic techniques as potential replacements of TLC for quantisation glutamine in TD drug substance and products.

HPLC with evaporative light scattering detection and LC/MS are also possible alternatives to the determination of glutamine. However, these methods are limited by the availability of these instruments in the laboratory. Indirect UV detection is attractive because such a method can be adapted easily for use with existing HPLC instruments. For these reasons, a more quantitative and automated HPLC method utilizing indirect UV detection was developed and validated for the determination of the non-UV absorbing glutamine in TD drug substance and product [39]. The HPLC mobile phases consisted of phosphoric acid, 2-naphthalenesulfonate sodium and methanol. 2-Naphthalenesulfonate was used as a UV detection probe for glutamine. A segmented isocratic elution program was used to elute glutamine and TD, respectively. The method was found to be specific for glutamine. The linearity was 0.05–1.25% glutamine with respect to a nominal concentration of 8 mg mL^{-1} TD sample. The limits of detection and quantitation were found to be 0.03% and 0.05% glutamine, respectively.

3. Other methods

3.1. Gas chromatography (GC)

Studies concerned with the stereochemical lability of TD and catalytic effects leading to enantiomerization appears as important as they were nearly 40 years ago. Enantioselective chromatographic methods, representing the most commonly used techniques for the determination of enantiomeric ratios, can also be used for the evaluation of stereochemical integrity. In this way, two chromatographic methods, dynamic GC and stopped-flow GC were used to determine the enantiomerization barrier of TD [40]. In the presence of a chiral stationary phase the enantiomers of TD produced characteristics elution profiles exhibiting plateaus and/or peak broadening which were observed between 190 and 220 °C in dynamic GC. To obtain the enantiomerization barrier of TD from experimental data, the fast and efficient simulation program ChromWin was used to simulate the elution profiles and obtain kinetic activation parameters.

3.2. Capillary electrophoresis (CE)

CE has been originally developed as a micro-analytical technique for the analysis of ionogenic compounds. Later micellar electrokinetic chromatography (MECC) allowed the analysis of neutral compounds. In 1989, enantioseparations of charged racemates were reported using cyclodextrins as chiral selectors in the free solution mode. The separation of some basic drugs and their metabolites and the resolution of enantiomers in the same run using CE are also described and the resolution of neutral drug enantiomers is possible by using charged chiral selectors in CE. In this way, Weinz and Blaschke investigate the biotransformation of TD [41]. The simultaneous enantioseparation of TD and two of its possible hydroxylated metabolites was achieved using CE with negatively charged carboxymethyl-β-cyclodextrin. This method was extended to one additional biotransformation product [42]. The dual chiral selector system using native β-cyclodextrin (β-CD) and the negatively charged sulfobutyl-β-CD was slightly modified up to a concentration of 12 mg mL^{-1} 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.

Enantioselective chromatographic methods, representing the most commonly used techniques for the determination of enantiomeric ratios, can also be used for the evaluation of stereochemical integrity. In a study, performed by Schoetz et al. [43], dynamic capillary electrokinetic chromatography (DEKC) was employed to determine the enantiomerization barrier of TD. In the presence of the chiral mobile phase additive carboxymethyl-β-cyclodextrin, the interconverting enantiomers of TD produced characteristic elution profiles exhibiting plateaus and/or peak broadening between 25 and 55 °C at pH 8. To obtain the enantiomerization barrier of TD from experimental data, the fast and efficient simulation program ChromWin was used to simulate the experimental interconversion profiles and to obtain the apparent rate constants.

Capillary electrochromatography (CEC) is a relatively new separation technique that combines the selectivity of LC with the high resolution and efficiency of CE. The advantages principally arise from the electroosmotic transport of mobile phase through the column which leads to reduced plate heights due to the plug-like flow and the ability to employ smaller particles and longer columns for even higher efficiency. These advantages of CEC have contributed to its rapid development, which has resulted in a diverse range of applications from small molecules to large proteins.

The separation selectivity and efficiency values that are possible in CEC thus make it an attractive complimentary technique to those available for the separation of enantiomers. For this reason, a vancomycin chiral stationary phase (CSP) was fully evaluated in CEC in reversed-phase and polar organic modes for a number of racemic pharmaceutical compounds. High efficiency and resolution values were obtained for a number of compound classes including TD in both the polar organic mode and reversed-phase mode [44]. The same investigation group

demonstrates the feasibility of preparing in situ a vancomycin CSP for CEC [45]. Initially capillary columns are packed with LiChrospher® diol silica and evaluated in an achiral mode to ensure adequate packing and retaining frit production. The CSP is subsequently prepared using a simple three-step reaction carried out by flushing the reagents for each step through the diol silica column. The in situ approach is adopted in preference to that of packing the well-characterised commercial phase or a batch synthesis primarily so that any observed enantioselectivity could be compared to earlier similar studies in LC and supercritical fluid chromatography (SFC) carried at our laboratories. The resulting vancomycin phase is evaluated in CEC for both the reversed-phase and polar organic mode with a number of neutral, basic and acidic racemic analytes.

3.3. Luminescence

The routine methodology employs a highly separation method (HPLC) with analyte detection based on molecular absorption in the UV region. A very sensitive phosphorimetric method has been developed, showing the analytical potential of the luminescent properties of TD [46]. The phosphorescence of TD was greatly improved using Hg(II) as a selective heavy atom enhancer in a low background cellulose substrate. Although very sensitive (1.8 ng absolute detection limit) and selective, this method is time consuming because the samples need to be dried and purged with N_2 in the sample holder for 5 min before each measurement. For this reason, a spectrofluorimetric method was developed and evaluated in terms of sensitivity and selectivity for determination of TD in pharmaceutical formulations, blood serum and urine [47]. Efforts were spent on the maximization of TD fluorescence signal and on the increasing of selectivity when analyzing complex samples, specially, in samples containing sulfanilide, a strongly fluorescent concomitant that can be used in association with TD. Maximum signal was observed with optimized composition of the solvent system and pH. Better results were achieved using the combined use of acidic medium and UV treatment. For urine and blood serum samples, a solid phase extraction on a C_{18} cartridge was found to be practical. Limit of detection was estimated to be 1.2 $\mu g\,L^{-1}$, approximately 100 times better than the ones reported for the routine UV absorption HPLC methods.

3.4. Voltammetry

Although the luminescence based methods are very sensitive and selective, the determination of TD often requires higher sensitivity because it suffers fast hydrolysis, decreasing analyte concentration that must be measured in biological fluids. Recently, Liu et al. in 2002 had investigated the electrochemical behaviour of TD in order to understand the nature of its reduction process on the mercury electrode. Although reporting experimental conditions for the accumulation of the analyte on the HMDE, but the authors have not developed an analytical method. Cardoso et al. [48] develop, evaluate and compare an adsorptive stripping differential-pulse voltammetry (DPV) and square-wave voltammetry (SWV) methods for ultra-trace determination of TD in pharmaceutical formulations and biological fluids (blood serum and urine). The most sensitive TD reduction peak at −804 mV for SWV and at −921 mV for DPV was used. Selective pre-concentration of TD on the static mercury drop electrode was achieved, increasing the sensitivity of determinations. Absolute limit of detection, for 100 μL sample of 4.7 pg for DPV and 0.5 pg for SWV.

3.5. Mass spectrometry

Significant progress has been made during the past years on methods of chiral identification and quantification based exclusively on mass spectrometry. In comparison with chromatographic methods, which are usually employed in quantitative chiral analysis, the kinetic method approach is faster and also requires smaller amounts of sample.

Augusti et al. [49] describe the application of the kinetic method to enantiomeric quantisation of TD; all experiments were performed using a commercial ion trap mass spectrometer equipped with an electrospray ionization (ESI) source and operated in the positive ion mode. The mass spectra reported are the average of about 40 scans, each requiring 0.2 s. Samples were infused into the ESI source via a syringe pump at a flow rate of 2.00 $\mu L\,min^{-1}$.

4. Conclusions

TD is used as a racemate with potentially different pharmacokinetics and pharmacodynamics of the component (+)-(*R*)- and (−)-(*S*)-TD enantiomers; moreover, these are biotransformed to a number of potentially active chiral and achiral metabolites. Many methods have been developed to quantify TD and its metabolites from a variety of biological matrices. Most of these methods are achiral, being based on HPLC using reverse phase separation with UV, MS, or tandem MS detection. However, increasing recognition of the potential importance of stereoselectivity in TD pharmacology has, more recently, led many investigators to separate its enantiomers, in some cases also with separation of various metabolites. Such approaches are commonly based on chiral stationary phase (CSP)–HPLC, CE or electrochromatography (CEC) technology. Also recently, vancomycin has been utilized as a CSP for separating a number of racemic drugs, including TD; the results showed that vancomycin HPLC–CSP column is a good choice for separation and quantification of TD enantiomers.

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