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Sensitive and rapid method for the determination of thalidomide in human plasma and semen using solid-phase extraction and liquid chromatography–tandem mass spectrometry

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Abstract

Liquid chromatography–tandem mass spectrometric assays were developed for the sensitive, rapid and high throughput bioanalyses of thalidomide in human plasma and semen. The matrices were first stabilized with 0.025 *M* Sorensen's citrate buffer at pH 1.5 to prevent spontaneous hydrolysis. Buffered thalidomide 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 an APCI interface in the negative ion mode. Calibration curves for both matrices were linear with $r>0.99$ from 2 to 250 ng/ml and ng/g. Inter-assay precision (RSD) of plasma and semen calibration standards were 2.6–11.6 and 1.9–12.4%, respectively. Recoveries from plasma and semen were greater than 69 and 78%, respectively. Batch sizes of 100 samples per matrix were analyzed with a total run time of 5 h. The methods successfully determined concentrations of thalidomide from a clinical study to levels as low as 7 ng/ml plasma and 8 ng/g semen, respectively.

Keywords: Thalidomide

1. Introduction

Thalidomide was used as a sedative and anti-emetic in Europe but was banned in the early 1960s when its teratogenicity became evident (Fig. 1). The US was spared the fetal tragedy through the diligence of an FDA reviewer, Frances Kelsey, who demanded more studies prior to approval [1]. The sponsoring company was not forthcoming and the drug was never approved. In 1998, Celgene obtained approval for thalidomide (THALOMID®) in the US for the treatment of erythema nodosum leprosum (ENL), a cutaneous manifestation of lepromatous leprosy [2]. Due to its history, the approval came with unprecedented restrictions to prevent potential fetal exposure [3]. Thalidomide is currently being investigated for activity in various oncologic, inflammatory and dermatologic conditions [2,4–6]. It

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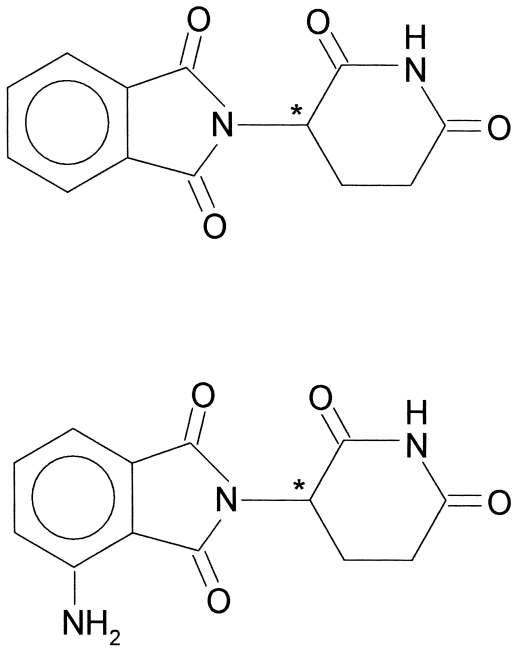

Fig. 1. Structures of thalidomide (top) and the internal standard (bottom). Asterisks denote chiral center.

recently showed significant activity in refractory multiple myeloma as well as reducing the gastrointestinal side-effects from irinotecan (CAMPTOSAR®) chemotherapy for metastatic colorectal cancer [5,6]. Thalidomide has also been found to possess potent anti-angiogenic activity giving rise to numerous clinical trials in oncology [4,7].

Thalidomide spontaneously hydrolyses in aqueous media at physiological pH [8]. Acidification has been shown to reduce hydrolysis thereby facilitating bioanalysis [9]. Over the years, numerous HPLC assays of racemic thalidomide in plasma and serum have been developed [9–16]. An LC–MS assay was recently described for the detection of thalidomide metabolites [17]. In this paper, we describe highly sensitive, rapid, selective and high throughput LC–MS–MS assays of thalidomide in human plasma and semen with calibration ranges of 2–250 ng/ml and ng/g, respectively. Both assays were developed and performed according to Good Laboratory Practice regulations [18] and acceptable industry standards [19].

2. Experimental

2.1. Chemicals

Thalidomide (α-phthalimidoglutarimide; MW 258; CAS #50-35-1) is a low water soluble white powder stable at room temperature. It was synthesized by Chemsyn Science Laboratories (Lenexa, KS) and its purity (100%) determined by HPLC [20]. A thalidomide analogue was used as the internal standard (Fig. 1). It had a purity of 98.8%. All chemicals used were reagent or HPLC grade. Plasma and semen was obtained in-house from a healthy volunteer.

2.2. Calibration, quality control, internal and system suitability standard solutions

Thalidomide and internal standard stock solutions were prepared in methanol and acetonitrile–methanol (50:50 v/v), respectively. A 500 μg/ml calibration and Quality Control (QC) stock solution was initially prepared. Working calibration and QC standards of 100–50 000 ng/ml were then prepared. Aliquots of the calibration and QC standards were spiked into matrices to give calibration curve and QC sample solutions of 2, 4, 10, 25, 50, 100, 200 and 250 and 2 (Lower Limit of Quantitation, LLOQ), 5 (Low QC), 125 (Medium QC), 190 (High QC), 250 (Upper Limit QC) and 1000 (Diluted QC) ng/ml for plasma and ng/g for semen. Diluted QC samples were analyzed after 5-fold dilution with buffered control matrix. A 500 μg/ml internal standard stock solution was prepared and used to make working internal standard solutions of 2500 and 50 000 and 250 and 5000 ng/ml for plasma and semen, respectively. System suitability standards of 2 and 250 ng/ml were prepared from calibration curve solutions and working internal standards using water–acetonitrile–acetic acid (90:10:0.1, v/v/v). All calibration and QC samples were immediately stabilized with 0.025 *M* Sorensen's citrate buffer pH 1.5 (50:50, v/v) to reduce hydrolysis [9]. Acidification also served to

precipitate plasma protein for cleaner extraction of thalidomide.

2.3. Sample extraction

Frozen samples were thawed to room temperature, mixed and centrifuged for 5 min at 3000 rpm at 10 °C. Internal standard (2500 ng/ml plasma; 250 ng/ml semen) was added to the appropriate samples. Oasis HLB SPE cartridges (Waters Chromatography, Watford, UK) were primed with methanol followed by 0.5% formic acid and then plasma and semen samples were passed through. Cartridges were then washed with water–methanol–formic acid (70:30:0.5 v/v/v) followed by water. Thalidomide was then eluted with methanol, evaporated under nitrogen, reconstituted in water–acetonitrile–acetic acid (90:10:0.1 v/v/v), centrifuged and the supernatant analyzed by LC–MS–MS.

2.4. Recovery assessment

2.4.1. Reference samples

Thalidomide reference samples were prepared from buffered control plasma and semen. They were extracted and spiked with thalidomide to yield Low, Medium and High QC samples. Extracts were similarly spiked with internal standard.

2.5. Stability

2.5.1. Room temperature

Buffered non-extracted control plasma and semen at Low, Medium and High QC concentrations were analyzed after being allowed to stand for 24 h at room temperature in condition similar for study samples.

2.5.2. Freeze–thaw

Buffered plasma and semen at Low, Medium and High QC concentrations were analyzed after three additional freeze–thaw cycles.

2.5.3. Sample extract

Plasma and semen QC sample extracts were stored refrigerated for 24 h. They were then injected together with freshly prepared sample to determine if any analyte was lost. Stability of extracted samples in the autosampler tray for duration of LC–MS–MS analysis was determined by comparing calculated concentrations of QC samples injected throughout the intra-assay batch.

2.6. LC–MS–MS

Bioanalysis was performed on a Hewlett-Packard HPLC system (G1312A 1100 series; Cheshire, UK) coupled to a Micromass Quattro LC tandem mass spectrometer (Manchester, UK) equipped with an APCI interface in the negative ion mode. The HPLC was fitted with a 4×4 mm C_{18} precolumn (Phenomenex, Cheshire, UK) connected to a 3.5 cm×3.2 mm Summit column containing 3 μm ODS C_{18} packing (Crawford Scientific, Lanarkshire, UK). The mobile phase was water–acetonitrile–acetic acid (75:25:0.1, v/v/v) at a flow-rate of 0.5 ml/min with no splitting. The source temperature was 150 °C and the corona voltage was 3 kV. Collision and nebulizer/desolvation gases were argon and nitrogen, respectively. Collision gas cell pressure was 3.5×10^{-4} and 1×10^{-3} mbar for plasma and semen, respectively. The desolvation gas flow was 200 l/h. Injection volumes were 50 and 75 μl for plasma and semen extracts, respectively.

2.7. Analysis of thalidomide in plasma and semen from HIV-seropositive patients

An 8-week double-blind, placebo-controlled, parallel group study with a 4-week off-treatment follow-up visit was performed in HIV-seropositive adult volunteers. They were given 100 mg of thalidomide/day and plasma and semen samples taken within 13 h after dosing at weeks 4, 8 and 4 weeks after cessation of dosing. A pre-dose sample was also taken. Heparinized plasma (0.5 ml) and semen (variable) samples were immediately stabilized with Sorensen's citrate buffer (v/v and w/w, respectively) and stored at −70 °C until thawed for assay. Bioanalyses were performed using the validated assays as described above.

3. Results

3.1. LC–MS–MS chromatograms

Representative LC–MS–MS profiles of thalidomide in plasma and semen are shown in Fig. 2.

3.2. Standard curves, precision and accuracy

Standard curves for four batches of plasma and semen were linear with regression equations of $Y = 0.0164x + 0.0017$ and $Y = 0.0215x + 0.0045$. Standard deviations for the slopes and intercepts were 0.00338, 0.00660 and 0.00351, 0.00763 for plasma and semen, respectively, with correlation coefficients of 0.9902 and 0.9989.

3.3. Plasma

Precision and accuracy were within acceptable limits. Intra- and inter-assay precision and accuracy of QC plasma samples are shown in Table 1. Inter-assay precision (RSD) of calibration standards were 11.6% at 2, 9.8% at 4, 8.6% at 10, 12.3% at 25, 5.9% at 50, 2.6% at 100, 4.6% at 200 and 6.1% at 250 ng/ml. Coefficients of determination of the calibration curves were between 0.9806 and 0.9999.

3.4. Semen

Precision and accuracy were within acceptable limits. Intra- and inter-assay precision and accuracy of QC semen samples are shown in Table 1. Inter-assay precision of calibration standards were 12.4% at 2, 7.3% at 4, 5.7% at 10, 9.7% at 25, 7.2% at 50,

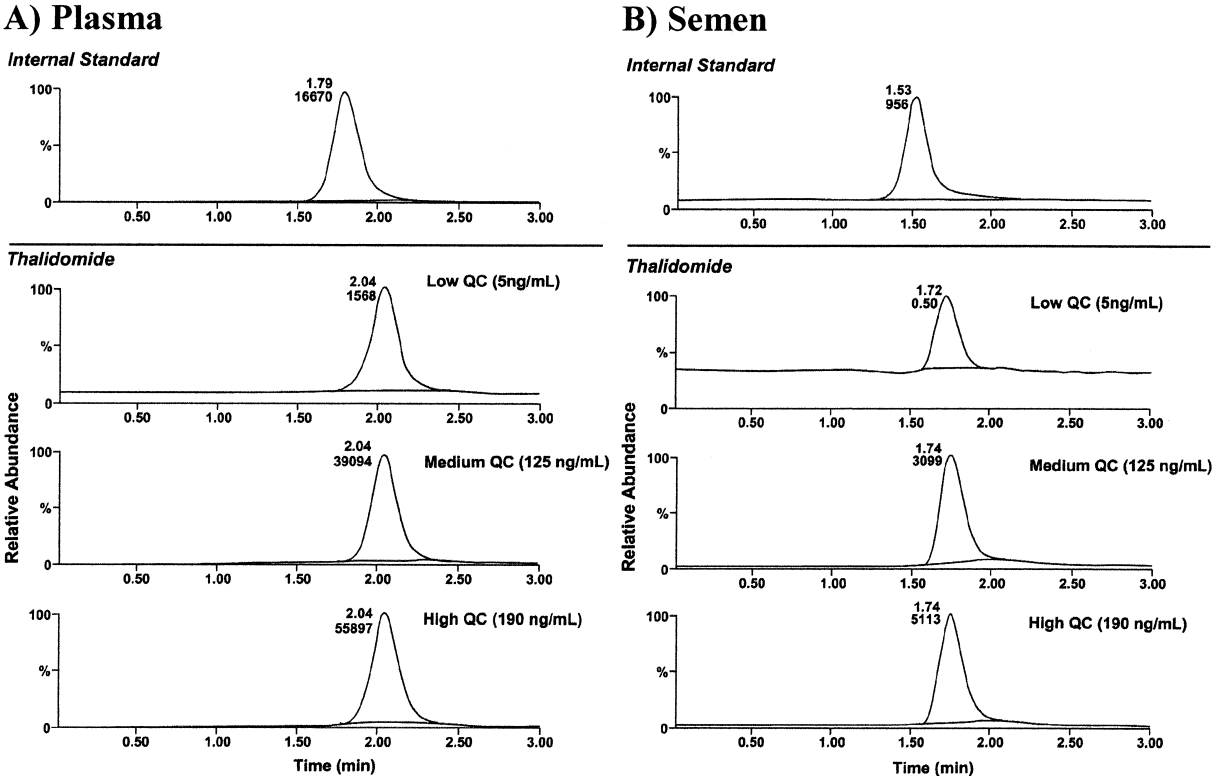

Fig. 2. Mass spectra of thalidomide in human plasma and semen over three QC concentrations.

Table 1
Intra and inter-assay precision and accuracy for the determination of thalidomide in human plasma and semen

QC (ng/ml or ng/g)	Plasma						Semen					
	Observed concentration (ng/ml)				Accuracy (%)		Observed concentration (ng/g)				Accuracy (%)	
	Intra	C.V. (%)	Inter	C.V. (%)	Intra	Inter	Intra	C.V. (%)	Inter	C.V. (%)	Intra	Inter
2	2.18±0.10	4.4	–	–	109.0	–	2.05±0.07	3.6	–		102.5	–
5	5.10±0.38	7.4	5.26±0.40	7.6	102.0	105.2	5.48±0.67	12.2	5.23± 0.80	15.4	112.3	107.2
125	114.71±3.34	2.9	127.84±9.05	7.1	91.8	102.3	134.22±14.76	11.0	125.76±10.54	8.4	110.0	103.1
190	182.17±16.28	8.9	201.80±18.14	9.0	95.9	106.2	187.51±5.33	2.8	182.93±10.55	5.8	101.1	98.6
250	237.43±7.44	3.1	–	–	95.0	–	249.61±7.75	3.1	–	–	102.3	–
1000	982.02±32.97	3.7	979.60±117.72	12.0	89.2	98.0	905.79±64.45	7.1	887.05±103.44	11.7	92.8	90.9

Observed concentrations are mean±SD (N=24 replicates).

4.5% at 100, 2.3% at 200 and 1.9% at 250 ng/ml. Coefficients of determination of the calibration curves were between 0.9978 and 0.9987.

3.5. Linearity, specificity, selectivity, recovery and carryover

The assay was linear from 2 to 250 ng/ml and ng/g for plasma and semen, respectively. No major interfering peaks in the reagent and matrix blanks were detected for thalidomide and the internal standard. Recovery of thalidomide from plasma at 5, 125 and 190 ng/ml and semen at 4.88, 122 and 185.44 ng/g were 80.7, 69.2 and 83.5% and 78.5, 81.4 and 85.0%, respectively. Mean recovery of the internal standard was 76.9 and 67.4% for plasma and semen, respectively. There was no carryover onto blank matrices after injection of High QC samples.

3.6. Room temperature, freeze–thaw and sample extract stability

Thalidomide was stable in buffered non-extracted plasma and semen samples when stored at room temperature for up to 24 h. Percent differences from baseline were −8.9, 2.7 and −1.4% and −3.8, −8.2 and −8.9% for Low, Medium and High plasma and semen QC concentrations, respectively. Thalidomide was also stable when exposed to up to three additional freeze–thaw cycles. Percent differences from baseline were −3.8, 4.8 and −2.7% and −8.8, −2.1 and −5.0% for Low, Medium and High plasma and semen QC concentrations, respectively. Thalidomide was stable in plasma and semen when refrigerated for 24 h. Percent differences from baseline were −1.0, 1.1 and 0.1% and −7.6, −14.4 and −6.3% for Low, Medium and High plasma and semen QC concentrations, respectively.

3.7. Batch size and analysis time

Batch sizes of up to 100 samples were analyzed with a total run time of 5 h each for plasma and semen. Run time for each sample was less than 5 min (Fig. 2).

3.8. Plasma and semen thalidomide levels from HIV-seropositive patients

Representative plasma and semen concentrations for four patients are shown in Fig. 3.

Two patients were unable to provide semen samples. Plasma and semen levels as low as 7 ng/ml and 8 ng/g, respectively, were successfully determined using the assays.

4. Discussion

There are currently numerous HPLC assays of thalidomide in plasma and serum with combined

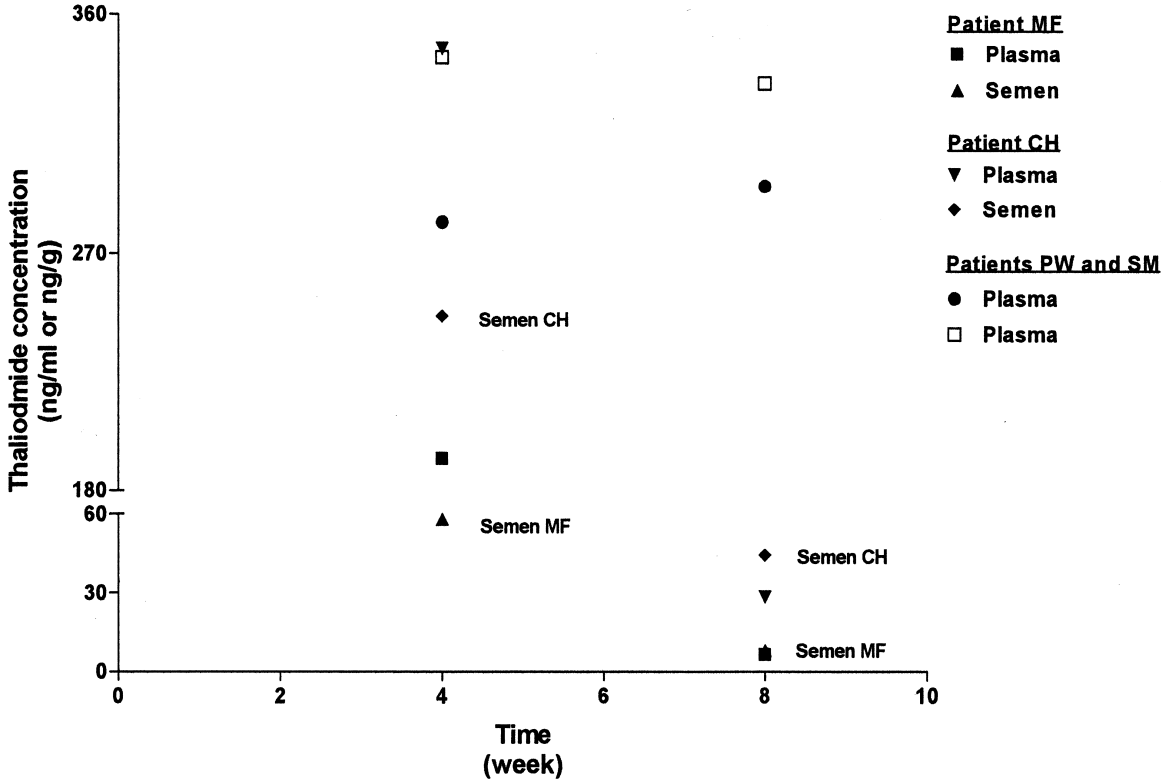

Fig. 3. Plasma (ng/ml) and semen (ng/g) thalidomide concentrations after daily oral administration of 100 mg/day for 8 weeks in selected HIV patients.

dynamic ranges of 0.06–22 μg/ml [9–16]. They generally use liquid–liquid extraction or protein precipitation followed by separation using reversed-phase columns and UV detection. We had earlier been using an HPLC method with liquid–liquid extraction and separation by a C_{18} column [16]. Accuracy was from 99 to 108%, precision was less than 4.5%, limit of detection was 0.1 μg/ml and linearity was 0.1–5 μg/ml. The method however used a high volume (1 ml) of plasma, generated liquid waste, had a 17 min run time and a high limit of detection. In addition, the increasing number of plasma samples from the over 150 ongoing clinical trials necessitated a rapid high-throughput thalidomide assay.

The decreasing cost of LC–tandem mass spectrometry instrumentation has enabled the development of highly selective and sensitive assays with rapid and high throughput capabilities. We describe LC–MS–MS assays for thalidomide in a common (plasma) and a rarely (semen) analyzed matrix. These assays were developed as part of a clinical trial of thalidomide in HIV patients. Plasma and semen thalidomide assays were successfully validated with no ensuing problems. The high selectivity of LC–MS–MS resulted in no interfering peaks in both assays. The limit of quantitation of 2 ng/ml for human plasma was significantly lower than the 0.06–1 μg/ml reported using UV detection [9–16]. This increased sensitivity and selectivity is achieved by using multiple reaction monitoring with the first and second quadrupoles monitoring the parent and daughter ions, respectively. Using the LC–MS–MS assay we were able to report on the presence of thalidomide in human semen after oral dosing [21]. Based on previous HPLC assays [9–16], it is unlikely that patients' thalidomide semen levels of below 100 ng/g could have been detected with great precision

and accuracy (Fig. 2). The wide linear range of the current assay should be adequate for plasma samples from the clinical trials. The lower plasma and semen levels at 8 weeks could be due to thalidomide inducing its own metabolism [22].

Recovery of thalidomide from plasma samples has been variable depending on the method used. The current method uses SPE cartridges for plasma clean-up which resulted in 70–85% recovery. The less than 90% recovery is probably due to some non-retention of thalidomide in the pre-elution and retention in the cartridge washes. Other investigators have reported recoveries of 80–95% [10,13,14]. This is comparable to the 80–85% recovery by protein precipitation [15]. Liquid–liquid extraction of thalidomide has generally yielded greater than 90% recovery [9,11,12]. This however is offset by the longer extraction time and generation of liquid waste. The current SPE clean-up method should be adequate for use with LC–MS–MS. Thalidomide had previously been shown to be stable in buffered non-extracted plasma [9]. Ancillary studies has shown that it is stable for up to 3 months when stored refrigerated at −70 °C (Celgene internal document). Our assay of patients used 0.5 ml of plasma. With the high sensitivity of LC–MS–MS, this volume could be scaled down considerably especially in patients with hematologic malignancies.

Thalidomide is mainly broken down through spontaneous hydrolysis [8,23,24]. It is still not known if the parent or one/more of the hydrolysis products cause the teratogenicity. The current assay could be modified to identify the over 100 putative hydrolysis products of thalidomide that are eliminated in the urine [8,23,24]. No problems were encountered in processing and analyzing the semen. Protein precipitation using Sorensen's buffer produced clean extracts and good recovery from semen. Similar results were obtained from the bioanalysis of thalidomide in rabbit milk (data not shown). In summary, we have developed sensitive, rapid and high-throughput LC–MS–MS assays of thalidomide in biological matrices. These assays will be helpful in pharmacokinetic evaluation of ongoing clinical trials.

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