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Development, validation and application of a sensitive LC–MS/MS method for the quantification of thalidomide in human serum, cells and cell culture medium

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ABSTRACT

A simple, robust, sensitive and selective liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the quantification of thalidomide was developed and validated. The method was applied to thalidomide quantification in three different types of biological samples. Thalidomide was extracted from human serum (100 μL), cells (2.5×10^5), or cell culture media (100 μL) by LLE and separated on a Prodigy C18 (150 mm × 4.0 mm, 5 μm i.d.) column with isocratic elution using water/acetonitrile (70/30, v/v) 0.1% formic acid, at a flow rate of 0.5 mL/min, with umbelliferone (600 ng/mL) as an internal standard. Thalidomide was quantified using a triple quadrupole mass spectrometer operated in multi-reaction-monitoring mode using positive electrospray ionisation. The method was validated in two separate thalidomide concentration ranges; human serum (0.05–20 μg/mL) and *in vitro* cells (0.78–50 ng) with an inter-day precision of 1.8% and 1.9% and average accuracy of 100% and 101% in serum and cells respectively. Despite the use of small sample volume, the limit of quantification for thalidomide in serum was determined to be 3 ng/mL. The method was successfully employed to measure levels of thalidomide in cancer patient serum and cell culture model systems. Although cellular levels were quantifiable, thalidomide was shown to be unstable under *in vitro* conditions with a half life of approximately 2 h. In patient samples, circulating serum levels showed a broad correlation with dose and uncovered some patient compliance issues.

1. Introduction

Thalidomide (α-N-phthalimido-glutarimide), was originally developed and marketed as an anti-morning sickness medication in the 1960 but was withdrawn following the proven associations with birth defects [1–3]. Thalidomide re-emerged in the clinic when its benefits to a patient suffering erythema nodosum leprosum (ENL), a potential complication of lepromatous leprosy, were discovered [4]. Subsequently, in 2006, a randomised trial by the Eastern Cooperative Oncology Group (ECOG) showed that a thalidomide–dexamethasone combination was more effective than dexamethasone alone in the treatment of multiple myeloma (MM). Based on this study, thalidomide was granted accelerated approval for the treatment of this disease by the FDA [5–7].

Teratogenicity, a major concern with thalidomide use, is managed by strict guidelines (STEPS programme [8]), however, the use of thalidomide in the treatment of MM commonly results is a variety of other adverse effects, the most common of which are constipation, neuropathy, somnolence, deep vein thrombosis (DVT) and depression [9].

Of the side effects reported, peripheral neuropathy is the most serious adverse effect associated with thalidomide treatment. Peripheral neuropathy is a sensation of tingling, pricking, or numbness (paraesthesia) in the fingers and toes, which, if left untreated can deteriorate into a serious and irreversible loss of sensory nervous function. To date no correlations have been made between patient serum levels, patient doses and the levels of neuropathy experienced. As a prelude to a larger pharmacotoxicological study, we sought to develop a method for the accurate quantification of thalidomide in the serum of MM patients to allow us to investigate potential correlations between circulating levels and toxicity.

Despite thalidomide being studied for several years, the *in vitro* analysis of thalidomide has yielded conflicting findings. Some groups have shown thalidomide activity *in vitro* [10] while other research reported that thalidomide inhibited activity *in vivo* but not *in vitro* [11]. At best, thalidomide has very poor potency in assays *in vitro* with researchers typically using unfeasibly high

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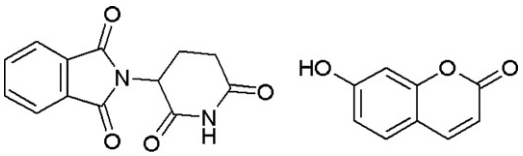

Fig. 1. (A) Thalidomide and (B) umbelliferone structure.

concentration to achieve results. Hence, in addition to measuring levels of drug in patient serum, we sought to broaden the applicability of our method to examine thalidomide levels *in vitro*.

Some of the original analytical methods developed for thalidomide focussed on the separation and analysis of stereoisomers of the agent [12]. The isomers have differing properties with a sedation effect primarily linked to the (*R*) isomer while the teratogenic effect has been linked to the (*S*) isomer [13,14]. However, thalidomide is administered as a racemic mixture and at physiological pH and temperature, these enantiomers rapidly interconvert [15]. Therefore, the separation of the enantiomers was unnecessary for our application. In analytical methods, thalidomide stability is also recognised as a problem but the majority of authors listed in Table 1 have addressed the issue of thalidomide hydrolysis through sample acidification prior to storage. A variety of sample clean-up methods have also been applied including SPE [16] and PPT [17–20] and LLE [21], though Saccomanni et al. [18] and Yang et al. [19] used the samples preparation method developed by Zhou et al. [20]. A summary of the method outlined in this research, and methods previously published in the literature for the analysis of thalidomide in biological matrices, are compiled in Table 1. Prior to the development of the method outlined here in, Teo et al. [16] describes the most sensitive and fastest assay, though with less than optimal recovery (68–79%). The method reported by Saccomanni et al. [18] gave the best recovery (>90%) while the adaptation of Yang et al. [19] reports similar recovery of >90%, though poorer sensitivity.

Developed here is a novel analytical method which is applicable to the sensitive quantification of thalidomide from a range of different biological matrices (serum, culture medium and cells) and uses a proportionately small amount of sample. The use of liquid–liquid extraction method gives a clean reproducible analyte extraction.

2. Experimental

2.1. Chemicals and solvents

Thalidomide and umbelliferone were purchased from Sigma–Aldrich, Dublin (Fig. 1). Water, acetonitrile (ACN) and formic acid, MS grade were purchased from Sigma–Aldrich, Dublin. Extraction solvents *tert*-butyl methyl ether (*t*BME), dichloromethane and ethyl acetate (HPLC grade) were purchased from Sigma–Aldrich, Dublin and Fischer Scientific, Dublin. Human serum (S7023) was purchased from Sigma–Aldrich, Dublin.

2.2. Standards and solutions

Primary stock solutions of thalidomide and umbelliferone were prepared at 1 mg/mL in ACN and stored at −20 °C. Working stock solutions were prepared fresh daily in acetonitrile to a concentration of 100 μg/mL of thalidomide and 10 μg/mL of umbelliferone. The internal standard (IS) was 600 ng/mL of umbelliferone. Citrate buffer, pH 1.5 was prepared as 25 mM sodium citrate in water, pH adjusted with HCl to 1.5. The extraction solvent was a mixture of ethyl acetate/ACN, 3:1 (v/v).

2.3. Patient samples

Twenty-one blood samples were collected from 11 MM patients. To combat the side effect of somnolence, patients are advised to take the thalidomide medication before bed and blood samples were collected in clinic the following morning. A blood sample of 10 mL was collected in additive-free blood tubes and the blood was allowed to clot for 30 min to 1 h. The non-clotted serum was transferred into a 15 mL Falcon tube and centrifuged at 400 × *g*, for 30 min at 4 °C. An aliquot of serum supernatant was mixed 1/1 (v/v) with 25 mM citrate buffer pH 1.5 in extraction tubes. Samples were frozen at −80 °C until extraction.

All blood samples were collected under the full ethical approval of the Mater Misericordiae University Hospital Ethics committee.

2.4. Cell samples

DLKP [25] and the drug resistant variant, DLKP-A [26], lung cancer cell-lines were cultured in DMEM (Dulbecco's Modified Eagle's Medium): Hams F12 50:50 supplemented with 5% fetal calf serum (Lonza). Accumulation and efflux assays were carried out as previously described [27] by seeding triplicate T25cm^2 flasks, allowing the cells to attach overnight and dosing the cells with 2 μM thalidomide for experimentally specified time points. After this, the media was removed and the cells were washed in cold PBS, trypsinised, and transferred to 10 mL polypropylene extraction tubes (Sarstedt). These were centrifuged at 200 × *g*, the waste media removed and resuspended in 1 mL of PBS – a small aliquot removed for cell count. The tubes were then centrifuged again, the supernatant removed and the cell pellet frozen at −20 °C in 50 μL 25 mM citrate buffer pH (1.5) for later extraction. The complete cell pellet was extracted according to the procedure outlined in Section 2.7.

2.5. Instrumentation

The chromatographic separation employed an Agilent (Ireland) 1200 Rapid Resolution LC system consisting of a degasser, binary pump, a thermostated column compartment and auto-sampler. Mass spectrometric detection was performed with an Agilent 6410 triple quadrupole system in multi reaction monitoring (MRM) mode interfaced with an electrospray ionisation source in positive mode.

A Labinco (The Netherlands) vortex, Stuart Scientific (UK) blood tube mixer and Thermo (Ireland) centrifuge were used during sample pre-treatment. A Genevac EZ-2 (Ipswich, UK) was used to evaporate solvent from extracted samples.

2.6. Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved using a Prodigy C18 column (150 mm × 4.0 mm i.d., 5 μm particle size) with a SecurityGuard C18 guard column (4 mm × 3.0 mm i.d.) both from Phenomenex, UK. A mixture of acetonitrile:water (30:70, v/v) 0.1% formic acid was used as mobile phase, at a flow rate of 0.5 mL/min. The column temperature was maintained at 20 °C and the temperature of the autosampler was maintained at 4 °C. The complete chromatographic run time of each sample was 13 min, which separated umbelliferone and thalidomide from each other with retention times of 7.9 and 9.3 min respectively (Fig. 2). System standards and quality control checks were run at intervals throughout the sample worklist. Peaks were quantified using Agilent Masshunter Software (Version B.01.04).

The mass spectrometer was operated using an ESI source in the positive ion detection mode. The ionisation temperature was 300 °C, gas flow rate was 11 L/min, nebuliser pressure was 345 kPa and the capillary voltage was maintained at 5000 V. Nitrogen was

Table 1
Comparison of analytical methods for thalidomide in biological matrices. ISTD – internal standard. The analytical method developed and validated here was summarised in first row for comparison.

Analyte, ISTD	Biological matrix	Sample clean-up	Sample storage	Analytical method	LC run time (min)	Sensitivity LOQ	% recovery	Author date ref
Thalidomide, umbelliferone	*Human serum (0.1 mL) Cell-line models*	*LLE*	*−80 °C, 1:1 (v/v) 25 mM citrate buffer pH1.5*	*LC–MS/MS*	*13*	*3 ng/mL*	*91.8–115.1%*	*Roche*
Thalidomide, phthalimide	Human plasma (1 mL)	LLE modified from Yang et al., 2005	−80 °C, 2/1 (v/v) in stability solution.	HPLC-UV	10	100 ng/mL	>90% within 0.05–50 μg/mL	Saccomanni 2008 [18]
Thalidomide, phenacetin	Human serum Rat serum and tissue (0.2 mL)	LLE	Serum 1/1 (v/v) tissue 1/4 (w/v) citrate–phosphate buffer (pH 2, 0.2 M), stored at −80 °C.	HPLC-UV	18	~25 ng/mL in serum	54–100% for enantiomers in different matrices	Murphy-Poulton 2006 [21]
Thalidomide, phenacetin	Rat plasma (0.1 mL)	200 μL acetonitrile/methanol (1:1, v/v) containing 2% (v/v) acetic acid	−20 °C	HPLC-UV	11	51.6 ng/mL[a]	>90%	Yang 2005[b] [19]
Thalidomide, phenacetin	Hanks balanced salt soln. (0.4 mL)		1/2 (v/v) ice-cold acetonitrile/methanol mixture containing 2% acetic acid (v/v)	HPLC-UV	10	6.4 ng/mL[c]	90–110%	Zhou 2003 [20]
Thalidomide	Plasma & semen (0.5 mL)	SPE	0.025 M Sörensen's citrate buffer pH 1.5, −70 °C	LC–MS–MS	4	5 ng/mL	Plasma > 69% Semen 78%	Teo 2002 [16]
Thalidomide, phenacetin	Human serum (0.5 mL)	Protein precipitation		HPLC-UV	>16	222 ng/mL	79–84%	Toraño 1999 [17]
Thalidomide enantiomers, labetalol	Plasma (1 mL)	SPE	1:1 (v/v) 0.025 M phosphate buffer, pH 2.5	HPLC-UV	13	Not specified	Not specified	Haque 1998 [12]
Thalidomide, phenacetin	Human serum (0.5 mL)		10% H_2SO_4 (7.4 μL) in 500 μL serum, −35 °C	HPLC-UV	20	50 ng/mL	Not specified	Simmons 1997 [22]
Thalidomide, ciprofloxin	Human plasma (1 mL)	SPE[d]		HPLC-UV	~20	62.5 ng/mL	79.5%	Delon 1995 [23]
Thalidomide, phenacetin	Rat plasma and blood (0.2–2 mL)	LLE	1:1 (v/v) 0.025 M Sörensen's citrate buffer, pH 1.5, −25 °C	HPLC-UV	>6	Not specified	Plasma 93% Blood 87%	Eriksson 1992 [24]

[a] Reported as 0.02 μM.
[b] Adapted from Zhou et al. [20].
[c] Reported as 0.32 ng/50 μL aliquot of thalidomide in HBSS.
[d] No reference is made to stabilising the samples to avoid hydrolytic degradation.

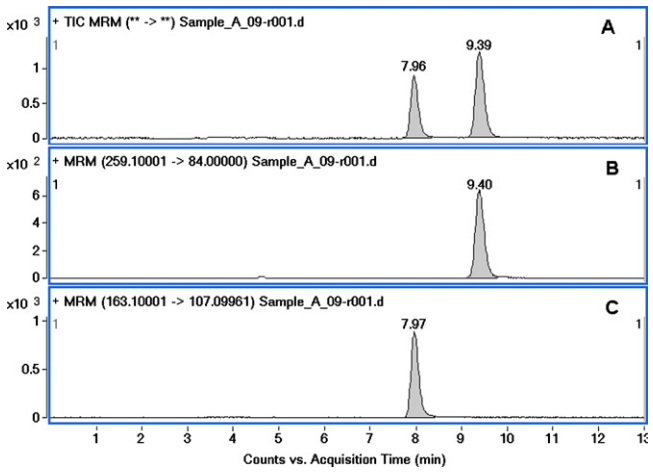

Fig. 2. Representative total ion current (TIC) chromatogram (A) and one extracted MRM chromatogram for thalidomide (B) and umbelliferone (C).

used as the ionisation source gas and ultrapure nitrogen as the collision cell gas.

Analysis was performed in MRM mode with the following transitions: m/z 259.1 → m/z (186 and 84) for thalidomide, and m/z 163.1 → m/z 107 for umbelliferone, with a dwell time of 200 ms. Both product ions of thalidomide were monitored, 84 m/z was the quantifier ion and 186 m/z was the qualifier ion. Table 2 details the transitions optimised for agents used where * indicates the quantifier ion.

2.7. Liquid–liquid extraction (LLE) procedure

For the extraction of serum samples and cell culture media, 200 μL of sample (serum: citrate 1:1, v/v or media: citrate 1/1, v/v) was added to a polypropylene extraction tube. For the extraction of cell samples, the total cell pellet/citrate buffer mixture was allowed to thaw in the extraction tube. To this, 50 μL of internal standard (600 ng/mL umbelliferone) was added, along with 50 μL of acetonitrile and 2 mL of extraction solvent (ethyl acetate/ACN, 3:1, v/v). The extraction tubes were vortexed and mixed on a blood tube mixer for 5 min. The samples were centrifuged at 3200 × *g* for 5 min. The 1.1 mL of the organic layer was removed with a glass Pasteur pipette and transferred to a conical bottomed glass LC autosampler vial (Chromacol). The vials were evaporated to dryness using a Genevac EZ-2 (Ipswich, UK) evaporator at ambient temperature, without light. The samples were reconstituted in 50 μL of mobile phase. Two injections of 20 μL were injected automatically by the autosampler.

2.8. Optimisation of sample pre-treatment

To determine the optimum system for liquid–liquid extraction a variety of extraction solvents and solvent mixes were examined. The immiscible solvents tested were: ethyl acetate (EA), dichloromethane (DCM), chloroform, chloro-1-butane, and *tert* butyl methyl ether (*t*BME), and combinations of these solvents with acetonitrile, results are outlined in Fig. 3. The effect of the stabilising citrate buffer on extraction efficiency was also examined.

Table 2
The optimal fragmentor voltages (FV) and collision energy (CE) settings.

Name	Precursor ion	Optimum FV	Product ion	Optimum CE
Thalidomide	259.1	90	84[a]	10
Thalidomide	259.1	90	186	20
Umbelliferone	163.1	120	107.1[a]	20

[a] Quantifier ion.

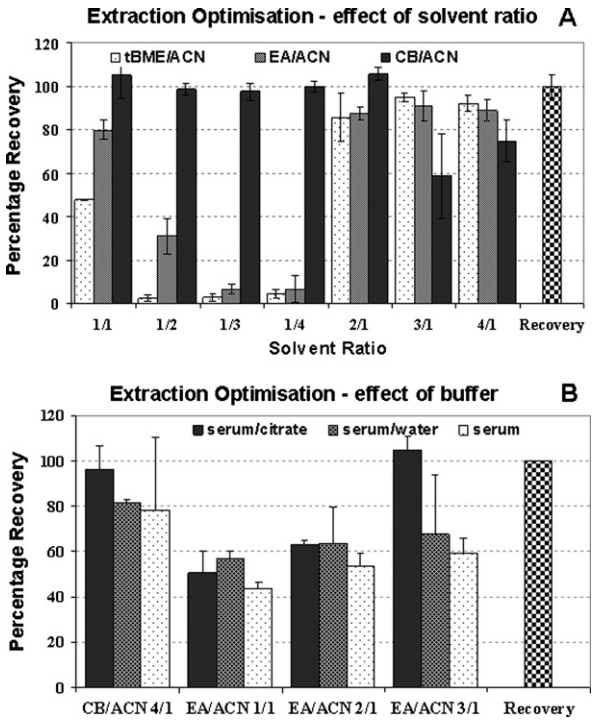

Fig. 3. The effect of varying the extraction solvent ratio on thalidomide recovery. Recovery was calculated based on the peak area of the extracted sample compared to the peak area of a pure drug calculated to be equal to 100% recovery. Data shown is the average of triplicate intra-day samples. tBME: tert-butyl methyl ether; EA: ethyl acetate; CB: chloro-1-butane. (B) The effect of the stabilising citrate buffer (25 mM, pH 1.5) on extraction efficiency of a selection of extraction solvent mixtures. The data shown is the average and standard deviation, $n = 6$.

2.9. Thalidomide validation samples

2.9.1. Serum validation – method 1

To 200 μL of matrix (human serum/citrate buffer, 1:1, v/v) 50 μL of internal standard (600 ng/mL umbelliferone) and 50 μL of analyte varying in concentration from 0.1 μg/mL to 40 μg/mL was added to an extraction tube, to give a serum concentration range of 0.05–20 μg/mL. These were extracted according to the method outlined in Section 2.7. All results and individual concentrations are detailed in Table 4A.

2.9.2. Cell validation – method 2

To a blank cell pellet, of approximately 2.5×10^5 cells, 50 μL of citrate buffer, 50 μL of internal standard (600 ng/mL umbelliferone) and 50 μL of analyte varying in concentration from 7.8 ng/mL to 1 μg/mL was added to an extraction tube, representing a spiked mass range 3.9–50 ng. As the quantification of thalidomide in cells is normalised to cell number, the mass of thalidomide rather than the concentration was used. The results are detailed in Table 4B.

2.9.3. Sensitivity

The sensitivity of the assay was monitored against human serum as a matrix. To determine the limits of detection and limits of quantification the concentration range was extended to a lower analyte concentration of 1 ng/mL which gives a serum concentration of 0.5 ng/mL. This extended the assay range below the LLOD and LLOQ to accurately confirm the LLOD and LLOQ.

2.9.4. Selectivity

The selectivity of the assay was assessed by spiking thalidomide (6.2–100 ng/mL) into human serum from three sources, one commercial and two patients. The patient samples were taken from MM patients who did not receive thalidomide as treatment. However, these patients were taking a range medication of co-administered similar to profile of MM patients receiving thalidomide. A low concentration range was assessed as any interference should be more pronounced at low concentrations.

2.9.5. Robustness

The development protocol identified the optimum conditions for the extraction and quantification of thalidomide in serum. Testing the robustness of the assay determines the effect of small changes in the procedure on the results. To this end a high, mid and low concentrations were spiked into human serum to examine minor variations in (1) the extraction solvent ratio (EA/ACN, 2.8/1 (v/v) and 3.2/1 (v/v)); (2) extraction solvent volume (1.9 mL and 2.1 mL); (3) the effect of citrate buffer pH on extraction (pH 1 and pH 2); and (4) the effect of serum volume on extraction (50 μL and 150 μL). The standard method was run as the control condition. As the serum volume was changed, the analysis was performed based on the spiked mass of thalidomide (0.5 ng, 50 ng and 2000 ng).

2.10. Statistical analysis

Where relevant measurements of experimental findings were statistically evaluated by means of standard deviations and two-tailed Student's *t*-test and a probability of $p < 0.05$ was regarded as a significant.

3. Results and discussion

3.1. Method development

3.1.1. Optimisation of chromatography conditions

Deuterated internal standards are commonly used in the quantification of analytes in bio-analytical chemistry though their cost frequently makes them prohibitively expensive. The use of deuterated internal standards is not always the best option for bio-analytics due to potential interactions with the analyte in terms of elution and extraction efficiency [28]. Umbelliferone has previously been used as an internal standard for lenalidomide, an analogue of thalidomide [29]. It is a cheap, easily available compound which is less toxic than phenacetin, the most commonly used internal standard for thalidomide.

A selection of chromatography columns was tested for the optimisation of the separation of thalidomide and umbelliferone. Given the use of an internal standard the application of an isocratic elution is the optimum as this provides continuous ionisation conditions. Optimum separation was achieved on the Phenomenex Prodigy C18 column (150 mm × 4.0 mm i.d., 5 μm particle size) with a mobile phase of water/acetonitrile/formic acid 30/70/0.1 (v/v) with isocratic elution (Fig. 2).

3.1.2. Optimisation of sample pre-treatment

Citrate buffer was added to the serum samples to stabilize the thalidomide and prevent spontaneous hydrolysis of thalidomide [30]. A variety of commonly used extraction solvents were initially examined to extract a known spiked concentration of thalidomide from serum/citrate mixtures. The peak areas of thalidomide extracted were compared to the peak areas of the calculated recovery samples. Fig. 3A and B shows some key results of the extraction optimisation procedure. Fig. 3A shows the effect of varying ratio of extraction solvents with ACN. In combination with ACN, the extraction efficiency of EA was greatly improved when EA was in surplus compared to ACN, *e.g.* recovery of 7% (EA/ACN 1/3, v/v) compared to 91% (EA/ACN 3/1, v/v). *Tert*-butyl methyl ether showed a similar trend as EA. Chloro-1-butane extraction results remained unaffected by the addition of ACN until the ratio was greater than 3/1 (v/v). Hence, the use of chloro-1-butane and ethyl acetate in combination with ACN as an extraction mixture was examined further.

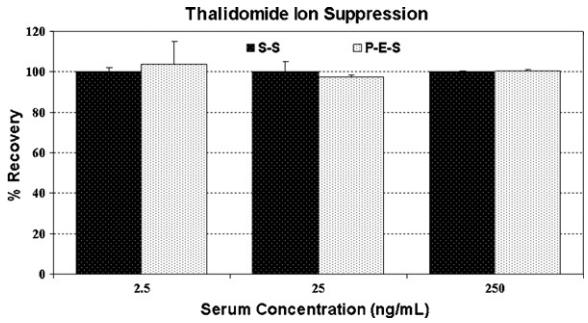

Fig. 4. The peak area of solvent standards was normalised to 100% and the post-extraction standards were expressed as a function of solvent standard. Data illustrates the mean and standard deviations of triplicate estimates where S-S indicates solvent standards and P-E-S indicates post extraction spiked samples.

Fig. 3B shows the effect of the acidification of serum with 25 mM citrate buffer pH 1.5 has on the extraction of thalidomide in a limited number of solvents. Two solvent mixtures showed potential as the optimum extraction solvent, ethyl acetate/acetonitrile (3/1, v/v) and chloro-1-butane/acetonitrile (4/1, v/v). The solvent mixture of ethyl acetate/acetonitrile (3/1, v/v) was determined to be the optimum extraction solvent as the recovery was consistent across a concentration range compared to choro-1-butane/ACN (4/1, v/v) (data not shown). Also the additional cost involved in the disposal of chlorinated solvents makes its use less desirable when alternative options are available.

3.1.3. Optimisation mass spectrometry settings

The optimum mass spectrometry settings (precursor ion, fragmentor voltage, collision energy, and product ion settings) for each analyte were identified by the separate flow injection analysis of 250 ng/mL of each analyte. The results are given in Table 2. To improve specificity, the MRM transitions defined in the optimisation stage were used. The most abundant product ion was used as the quantifier ion while any additional product ions were used as qualifier ions.

3.1.4. Ion suppression

The potential impact of ion suppression on the quantification was assessed by comparison of the results obtained with standard dilutions of each drug in acetonitrile (solvent standards) against standard dilutions which were added to dried extracted sample blanks (extraction standards), as outline by Zirrolli et al. [31].

Ion suppression was calculated as peak area of the analyte of interest in the post-extraction standard compared to the solvent standard. The findings are outlined in Fig. 4. Under the conditions tested, ion suppression effects were found to be minimal with no significant differences identified between the solvent standard (S-S) and post-extraction spiked (P-E-S) sample. A two-tailed Student's *t*-test was used to compare P-E-S to SS. The comparison did not find any significant difference between the pure solvent and extracted values ($p = 0.36$).

3.2. Method validation

The overall LC–MS method was validated for the following performance parameters – linearity and range, intra-day precision

(repeatability) and inter-day precision (intermediate precision), accuracy, sensitivity (LOD and LOQ), recovery and sample stability according to the guidelines described by Ermer [32], based on ICH and FDA guidelines [33]. MRM allowed individual determination of each drug necessary with the use of internal standard-based quantification.

3.2.1. Linearity and range

Regression analysis was used to assess the linearity between the peak area ratios (analyte/IS) and the analyte concentration.

The calibration curve for method 1, thalidomide in serum, was linear over the concentration ranges examined, serum concentration range 0.05–20 μg/mL. The calibration curve for method 2, thalidomide in cells, over the mass range of 3.9–50 ng in tube was also linear.

Since thalidomide and umbelliferone resolved well from each other and were extracted with high efficiency, umbelliferone was used as the internal analytical standard for thalidomide to reduce error. Whilst the calibration curves were linear, over such a broad range, the bias of the regression line tends to make the determination of lower drug concentration values much less accurate; hence a log–log plot of the peak area ratio *versus* the mass of drug was employed in all calculations. Typical correlation coefficients (R^2) values of >0.999 were seen in the standard curves of thalidomide extracted from both serum and cells across the validated ranges. More detailed information on the validation calibration curves are given in Table 3.

3.2.2. Precision and accuracy

For serum samples the intra-day precision and accuracy was assessed over a concentration range (0.05–20 μg/mL) by extraction and analysis of five spiked samples on the same day, while the inter-day precision and accuracy was assessed over the same concentration range of triplicate spiked samples over five days.

For cell samples the intra-day precision and accuracy was assessed over the in-tube mass range (0.39–50 ng) by extraction and analysis of six spiked samples on the same day, while the inter-day precision and accuracy was assessed on triplicate spiked samples over four days. The percentage relative standard deviation (%R.S.D.) was employed as a measure of precision. The percentage accuracy was determined by dividing the average calculated drug concentration by that of the spiked known concentration.

For serum sample validation the precision (%R.S.D.) in all cases was less than 5%, with the average %R.S.D. for the inter-day analysis being 1.8%. The accuracy of the assay ranged from 91% to 107%, with an average of 100% for the intra-day analysis and 99.8% average for the inter-day analysis, complete results detailed in Table 4A.

For the cell sample validation the intra-day analysis the average accuracy was 100% with an average %R.S.D. of 2.5%. The inter-day average accuracy was 101% with an average %R.S.D. of 1.9% complete results detailed in Table 4B.

3.2.3. Recovery/extraction efficiency

The extraction efficiency of the procedure was determined by comparing the peak areas of the extracted analytes with those from non-extracted (calculated) samples and samples extracted with no matrix present. Recoveries were evaluated across the concentration range. Recoveries for thalidomide drugs were generally good, with lower levels showing a slightly high percentage recovery. Results are shown in Tables 5A and 5B.

3.2.4. Sensitivity

The lower limit of detection (LLOD) was defined as the mass of drug which gave a signal to noise ratio of 3:1. The lower limit of quantification (LLOQ) was defined as the mass of drug which gave a signal to noise ratio of 5:1 [34]. The signal-to-noise ratio was calculated by the Masshunter Quantification Analysis Software (Version B.01.04). Calculations were based on the peak area of thalidomide, not on the peak area ratio.

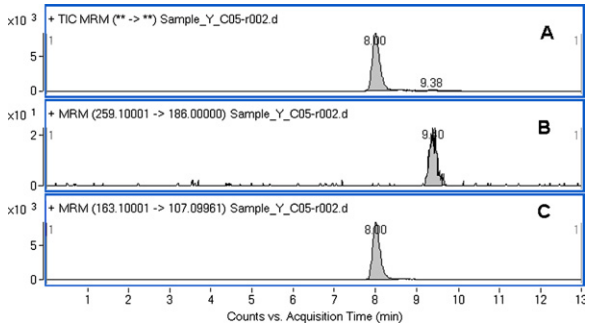

Fig. 5. Thalidomide LLOQ – 3 ng/mL TIC (A), extracted thalidomide MRM at 3 ng/mL (B), and extracted umbelliferone (ISTD) MRM (C).

Given the criteria outlined, the LLOD for thalidomide was determined to be approximately 1.6 ng/mL in serum, and the LLOQ for thalidomide was determined to be 3.1 ng/mL in serum (Table 6).

As the LLOQ was below the range needed for our analysis, this concentration was not included in the full validation. However, a serum concentration range of 3.1–50 ng/mL was examined in three different serum sources, one from a commercial supplier (Sigma) and the other two from MM patients not receiving thalidomide treatment. Table 7 gives the precision and accuracy of this analysis and Fig. 5 shows the extracted MRM transition of thalidomide at 3.13 ng/mL.

3.2.5. Selectivity

Thalidomide is often co-administered to MM patients with steroids (*e.g.* dexamethasone) and/or chemotherapy drugs (*e.g.* vincristine, doxorubicin) [35]. In this study thalidomide was administered in combination with dexamethasone (TD), in combination with melphalan, and prednisone (MPT) or in combination with cyclophosphamide and dexamethasone (CTD). The median age for diagnosis of MM is 68 year [36] and as such this patient population is prone to polypharmacy. The range of additional medication includes pain relief, anti-coagulants and laxatives to treat adverse effects as well as cardiovascular medication, anti-diabetic

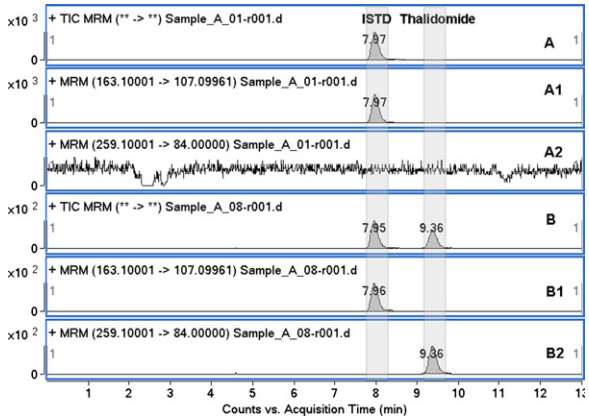

Fig. 6. Total ion chromatogram (TIC) and extracted MRM of two patient samples. A1 and A2 are extracted MRM transitions for umbelliferone and thalidomide respectively. These are extracted from the TIC A which is a sample from a MM patient not receiving thalidomide. B1 and B2 are extracted MRM transitions for umbelliferone and thalidomide respectively, extracted from the TIC B from a MM patient receiving thalidomide, where the thalidomide serum level was quantified to be 209 ng/mL. Comparison of A2 and B2 shows that there are no interfering peaks at the thalidomide elution time.

Table 3
Validation calibration curves information.

	Average slope (m)	% R.S.D.	Average intercept (c)	% R.S.D.	Average R^2	% R.S.D.
Method 1Serum[a]	1.06004	0.656	1.07584	5.189	0.9992	0.026
Method 2 Cells[b]	1.00625	0.522	2.07525	1.643	0.9996	0.008

[a] $n = 5$.
[b] $n = 4$.

Table 4A
Intra-day ($n = 5$) and inter-day ($n = 5$) precision and accuracy.

Method 1 – serum	Intra-day analysis			Inter-day analysis		
Spiked serum conc. (μg/mL)	Mean conc. observed (μg/mL)	Mean accuracy observed (%)	Precision (% R.S.D.)	Mean observed conc. (μg/mL)	Mean accuracy observed (%)	Precision (% R.S.D.)
20	18.2	91.2	4.1	18.2	91.2	1.8
10	10.1	100.9	2.3	9.9	99.2	2.2
5	5.3	106.8	0.7	5.2	104	2.4
1	1.03	103.3	4.5	1.1	106.3	1.6
0.5	0.53	106.5	3.9	0.52	103.2	1.1
0.1	0.1	98.6	4.2	0.1	99.1	2
0.05	0.05	98.2	1.7	0.05	95.7	1.8

Table 4B
Intra-day ($n = 6$) and inter-day ($n = 4$) precision and accuracy.

Method 2 – cells	Intra-day analysis			Inter-day analysis		
Spiked mass (ng)	Mean mass observed (ng)	Mean accuracy observed (%)	Precision (% R.S.D.)	Mean mass observed (ng)	Mean accuracy observed (%)	Precision (% R.S.D.)
50	50	99.4	0.8	50	100.8	1.5
25	25	100.5	1.2	25	100.8	0.9
12.5	12.5	100.4	1.8	12.5	100.3	1.9
6.25	6.24	99.9	1.6	6.3	100.5	0.7
3.13	3.09	98.9	2.8	3.1	99.6	1.1
1.56	1.58	100.8	3.3	1.6	100.6	2.5
0.78	0.79	100.6	4.0	0.8	101.5	2.4

Table 5A
Thalidomide intra-assay recovery ($n = 3$) in serum.

Method 1 – serum	Sample/calculated		Sample/no matrix	
Serum conc. (μg/mL)	Mean % recovery	% R.S.D.	Mean % recovery	% R.S.D.
20	108.4	7.3	93.1	6.3
10	127.5	5.3	99.2	4.1
5	113.4	5.4	97.7	4.7
1	109.5	4.9	98.8	4.4
0.5	120.3	5.9	97.9	4.8
0.1	120.0	6.1	93.5	4.8
0.05	126.4	4.3	95.1	3.3

Table 5B
Thalidomide intra-assay recovery ($n = 3$) in cells.

Method 2 – cells	Sample/calculated		Sample/no matrix	
Spiked mass (ng)	Mean % recovery	% R.S.D.	Mean % recovery	% R.S.D.
50	92.7	5.5	111.8	5.4
5	90.7	3.6	115.1	3.6
0.5	92.1	9.7	118.4	9.7

medication, arthritis medication among others for pre-existing conditions [37]. Therefore, to examine the selectivity of the method it was more applicable to spike serum from MM patients not receiving thalidomide but who were receiving a similar range of medication as those who receive thalidomide. The serum concentration range examined was close to the LLOQ as this is where any inferences from co-eluting substances would be most pronounced. Table 8 shows that when compared to the commercially available

Table 6
Thalidomide LLOD and LLOQ in serum ($n = 3$).

Serum conc. (ng/mL)	Mean conc. observed (ng/mL)	Mean accuracy observed (%)	Precision (% R.S.D.)	Mean signal/noise
3.1	3.2	103.8	2.0	6
1.6	1.6	100.4	1.2	4
0.8	0.8	97.8	2.8	2

Table 7
Intra-day accuracy and precision of low concentration thalidomide, demonstrating sensitivity, where matrix 1 indicates the commercially available serum and matrix 2 and 3 indicate serum samples from patients.

Serum conc. (ng/mL)	Matrix 1			Matrix 2			Matrix 3		
	Mean conc. observed (ng/mL)	% R.S.D.	Mean accuracy observed (%)	Mean conc. observed (ng/mL)	% R.S.D.	Mean accuracy observed (%)	Mean conc. observed (ng/mL)	% R.S.D.	Mean accuracy observed (%)
50.0	49.8	2.9	99.6	50.8	3.9	101.7	50.5	2.1	101.0
25.0	24.9	0.6	99.7	24.7	3.3	98.7	24.9	3.3	99.6
12.5	–	–	–	12.3	3.9	98.1	12.3	3.0	98.1
6.3	6.3	2.1	100.1	6.3	5.7	100.4	6.3	2.6	100.9
3.1	3.1	5.4	99.6	3.2	6.6	101.7	3.2	3.9	100.7

Table 8
the accuracy at low concentrations when using matrix from MM patients (2 and 3) as a matrix compared to a commercial matrix (1).

Serum conc. (ng/mL)	Matrix 1 Mean accuracy observed (%)	Matrix 2 Mean accuracy observed (%)	Matrix 3 Mean accuracy observed (%)
50.0	99.6	101.6	101.0
25.0	99.7	98.7	99.6
12.5	–	98.1	98.1
6.25	100.1	100.4	100.9
3.1	99.6	101.7	100.7

drug free matrix, the patient matrix had excellent accuracy, with an average accuracy across the concentration range for both patient samples of 100.1%.

The selectivity of the assay is increased through the use of MRM transitions for quantification. Fig. 6 shows the selectivity of the assay by comparing the TIC and extracted MRM of two patient samples, one patient who received thalidomide treatment (A) and a second patient (B) who did not, though was receiving other medication. This figure shows that no endogenous peaks were detected at the thalidomide retention time of 9.3 min in a sample from MM patients who was not receiving thalidomide (A2), indicating the selectivity of the assay.

3.2.6. Robustness

The robustness of the assay is a determination of the effect of small changes in the method on the method efficiency. For a method to be robust, it must be capable of withstanding small changes in the analytical procedure. Using a matrix design, we examined the impact of small variations in a number of parameters of the method at high, mid and low spiked masses. Data was correlated as spiked mass to better examine the effect of serum volume. As is seen in Fig. 7, small changes in the analytical procedure in terms of extraction solvent ratio or volume, buffer pH or serum volume had little or no effect on the quantified mass, indicating the method is robust. The validated method conditions were run as per the control conditions.

3.2.7. Stability

The stability of thalidomide in serum has been described previously [16,20,30,38] highlighting the importance of prompt storage of acidified thalidomide below −20 °C. Upon arrival in the lab, all samples were processed promptly, acidified with 25 mM citrate buffer, pH 1.5 and stored at −80 °C. The stability of thalidomide in serum was determined over four freeze–thaw cycles at serum concentrations of 0.05, 0.5, 5 and 20 μg/mL, as shown in Table 9.

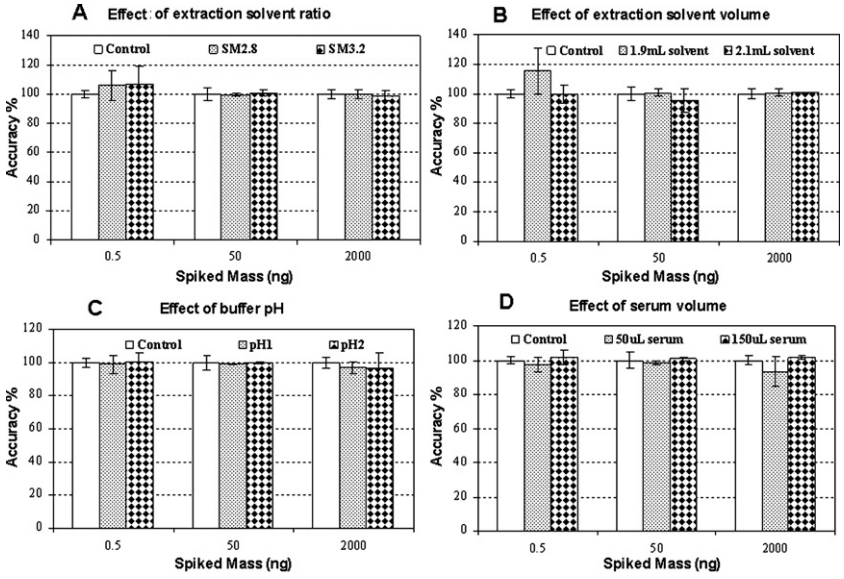

Fig. 7. Demonstration of assay robustness. (A) Extraction solvent ratio where SM2.8 indicated EA/ACN, 2.8/1 (v/v) while SM3.2 indicates a solvent ratio 3.2/1 (v/v); (B) extraction solvent volume 1.9 mL and 2.1 mL; (C) of citrate buffer pH at pH 1 and pH 2; (D) serum volume on extraction (50 (L and 150 (L). The validated method conditions were run as the control conditions.

Table 9
Thalidomide freeze/thaw stability in serum assessed by comparing the recovery of thalidomide in serum samples following four freeze/thaw cycles. All spiked serum samples were stored 1/1 (v/v) with citrate buffer. The data is the average of duplicate injections of triplicate samples ($n = 3$, $+n = 2$).

Serum conc. (μg/mL)	Day 0		Day 1		Day 2		Day 3		Day 4	
	% recovery	% R.S.D.	% recovery	% R.S.D.	% recovery	% R.S.D.	% recovery	% R.S.D.	% recovery	% R.S.D.
20	100	8.6	100	8.6	80	26.0	87	5.4	74^{+}	11.3
5	100	5.9	102	10.1	101^{+}	14.7	90	12.4	73	10.5
0.5	100	7.6	94^{+}	10.8	93	7.7	92	5.1	73^{+}	8.3
0.05	100	9.0	92	19.5	94	7.3	92	7.2	68^{+}	15.2

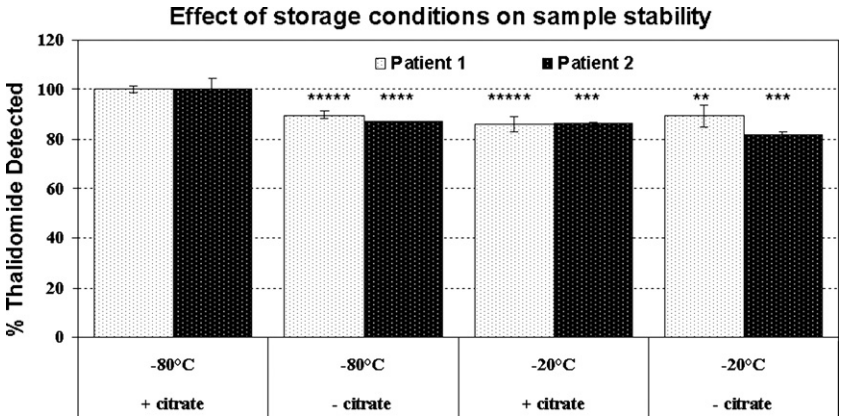

Fig. 8. Two independent patient samples were stored for 40 days prior to extraction and analysis. Standard storage conditions of −80 °C with citrate buffer was normalised to 100% and alternate conditions were expressed as a function of the routine storage condition. Significance was determined by Student's *t*-test, two-tailed where *>0.05, **>0.001, ***0.005, ****0.0001, and *****>0.0005.

The effect of storage conditions was also assessed in two independent patient samples. Serum was stored as prescribed in the protocol with citrate buffer at −80 °C. Serum from the same patients were also stored without citrate buffer and at −20 °C to examine the effect of alternative storage conditions for 40 days prior to extraction and analysis. Results shown in Fig. 8 demonstrate that thalidomide is most stable when stored −80 °C in citrate buffer. When the standard conditions (with citrate buffer pH 1.5 at −80 °C) were set as 100% and all other conditions were compared to it, a significant difference was seen with each alternative storage condition. Significance was determined by two-tailed Student's *t*-test, shown in Fig. 8.

3.3. Method application

3.3.1. Analysis of patient serum

The method developed was used to analyse 20 samples accrued from 11 MM patients, in a pilot study in collaboration with Mater Misericordiae University Hospital, Dublin. The thalidomide levels quantified in patient serum ranged from 25 ng/mL to 1407 ng/mL. The prescribed thalidomide dose of the patients ranged from 50 mg/day to 300 mg/day. The results showed that the administered dose broadly, but not directly, correlated with the detected thalidomide serum levels. Fig. 9 shows the correlation of patient dose and detected serum drug level. This agrees with previous study which showed that patients on low dose thalidomide (200 mg) had a lower C_{max} than patients on higher dose (800 mg) [39]. Our findings also indicate that although there is a broad correlation between dose and serum thalidomide level, there are significant inter-patient differences in circulating levels. This is not unexpectedly given that unlike many other chemotherapeutics, thalidomide doses are not normalised to patient variables such as weight or body surface area. To the best of our knowledge this in the only study which has shown such a dynamic intra-patient variability of serum concentration at a specific time point. This could possibly be due to patient variability in dosing schedules. The patients involved were sampled after different lengths of time on thalidomide and also the period of time between oral administration of thalidomide and blood sampling could have varied by a few hours among the population.

Also identified was some patient non-compliance with the oral medication instructions, as indicated in Fig. 9 by a circle. The indicated sample was one of three serum samples collected from one patient who was receiving a constant dose of thalidomide. The circled sample indicates that some doses were missed prior to one serum sample being taken, resulting in an unusually low thalidomide serum concentration.

MM is an incurable disease and thalidomide is playing an evolving role in the treatment of MM. For example, in the UK, the combination of cyclophosphamide/thalidomide/dexamethasone (CTD) is used as the up-front treatment of MM. A study by van Rhee et al., showed that patients with an initial higher cumulative dose of thalidomide had superior overall and event-free survival [40]. Recent studies of lenalidomide, an alternative MM

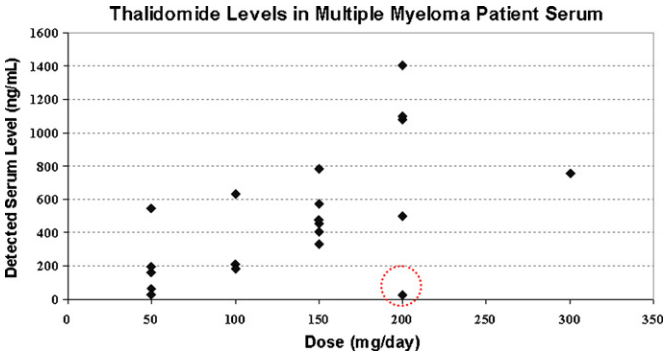

Fig. 9. Thalidomide analysis showing the correlation between administered thalidomide dose and quantified serum levels in 20 samples collected from 11 MM patients. The marked point indicated (on retrospective analysis) patient non-compliance.

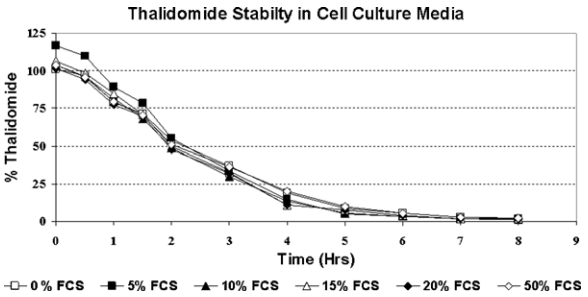

Fig. 10. Assessment of thalidomide's rapid degradation in cell culture media at 37 °C with 50% degradation observed after 2 h. The % FCS added to the media had no effect on thalidomide stability.

treatment, presented at the Annual Meeting of the American Society of Haematology (ASH) have suggested an increased risk of secondary cancers following lenalidomide treatment. This is currently being investigated [41,42]. Altogether, this demonstrates the need for an improved accurate analytical methodology to examine thalidomide pharmacokinetics to establish an appropriate treatment and maintenance schemes.

3.3.2. Analysis of cell samples and thalidomide stability in vitro

To date, the *in vitro* use of thalidomide has yielded conflicting results with the cellular pharmacology of thalidomide being poorly understood. Therefore, using a clinical relevant concentration (2 μM, 0.5 μg/mL), we attempted to quantify thalidomide in cell-line models with a series of efflux and accumulation assays of the agent at different concentrations and time points after drug exposure. The samples were stored and extracted as described in Section 2.7. Of the 116 samples analysed, thalidomide was detected in 16 samples, the detected cell levels ranging from 0.5 ng/million cells to 46 ng/million cells and no treatment-correlated alterations in levels were evident. This indicates that while the extraction is a stable and robust analytical technique, as shown through the validation procedure, in *in vitro* assays thalidomide, in an aqueous environment at 37 °C and neutral pH, is unstable at clinically relevant concentrations.

Previously, Zhou et al. [20] examined thalidomide stability in Hank's Balanced Salt Solution (HBSS) for the determination of thalidomide transport *in vitro* and found that pH had an effect on thalidomide stability in HBSS. Using method 1, we examined the stability of thalidomide in cell culture media. This showed that thalidomide is highly unstable in cell culture media at neutral pH, after 2 h thalidomide degradation was such that only 50% of the administered amount was found (Fig. 10). This is similar to previous findings by Eriksson et al., which showed degradation half-life of 4 h at 37 °C [24,43].

The exact mechanism of thalidomide action *in vitro* and *in vivo* remains poorly understood. It has been shown through *in vitro* techniques that thalidomide inhibits the effect of TNF-α, though all assays used high (μg/mL) concentrations of thalidomide in the assay [44–46]. Other groups have reported *in vivo* results that were not seen *in vitro* [11]. Thalidomide is noticeably inactive *in vitro* with researchers using pharmacologically unfeasible concentration to achieve results. Our results suggest this is probably due to instability of the drug under cell culture conditions.

4. Conclusions

A novel, robust, sensitive and broadly applicable LC–MS based assay has been developed and validated for the determination of thalidomide levels in a variety of biological matrices ranging from human serum to cancer cell lines. The use of a liquid–liquid extraction clean up stage gives the simplicity of application making the method accessible to other researchers undertaking large numbers of analyses without the need for extra equipment or clean-up columns. An isocratic elution scheme gives a simple, robust and reproducible chromatographic separation.

The method developed and validated here is more sensitive than previously published methods. This increased level of sensitivity afforded by an efficient sample preparation technique and sensitive MS/MS detection allows for the use of small samples volumes. This method was applied to the quantification of thalidomide serum levels in MM patient samples where a broad correlation was evident between dose and circulating level. Given the evolving role of thalidomide in MM treatment, further studies of serum levels to establish appropriate and safe maintenance regimes will be required. This method is also applicable to the determination of incidences of non-compliance in patients.

The applicability of the analytical technique was also extended to the quantification of thalidomide in *in vitro* assays. Cellular levels were very variable and our evidence suggests this is due to the rapid degradation of the drug in *in vitro* assay conditions, with 50% degradation in 2 h, a finding that may have significant implications in the interpretation of published *in vitro* thalidomide experiments and design of future studies.

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