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Thalidomide enantiomers: Determination in biological samples by HPLC and vancomycin-CSP

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Abstract

Thalidomide is a racemate with potentially different pharmacokinetics and pharmacodynamics of the component (+)-(*R*)- and (−)-(*S*)-thalidomide enantiomers. As part of a project on the adjunctive effects of thalidomide and cytotoxic agents, a method for the chiral separation and quantitation of thalidomide was developed and validated. Thalidomide 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 thalidomide 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. Over a thalidomide concentration range of 0.1–20 μg/ml, assay precision was 1–5% (CV) for both enantiomers, and calibration curves were linear with all correlation coefficients being >0.99. The estimated limit of quantification for both enantiomers was 0.05 μg/ml with 0.2–0.6 ml serum samples. Thalidomide 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 thalidomide treatment for mesothelioma, and to serum, blood and tissue samples from a laboratory rodent model using transplanted 9 l gliosarcoma. Enantioselectivity in thalidomide pharmacokinetics has been found, thereby reinforcing the need for considering the relevance of chirality in thalidomide pharmacology.

Keywords: Thalidomide stereoisomers; Enantioselective pharmacology; Cancer models; Pharmacokinetics

1. Introduction

Recent studies have focused on activity of thalidomide in glioma, a condition in which standard treatment remains disappointing, due in part to the poor penetration of many cytotoxic agents through the blood brain barrier. A clinical trial at the Royal North Shore Hospital of the anti-angiogenic agent thalidomide as treatment for relapsed glioblastoma multiforme (GBM) revealed a greater than 40% stabilization of disease [1], and others have reported the results of a phase II trial of thalidomide also suggesting useful activity [2]. The mechanism of action of thalidomide in glioma has not yet been fully elucidated and is the subject of ongoing research in our institution. Current data indicate that thalidomide has anti-angiogenic activity [3], and it has been hypothesised that thalidomide and chemotherapy might synergise, perhaps due to pharmacodynamic and/or pharmacokinetic interactions [4].

Thalidomide is used as a racemate with potentially different pharmacokinetics and pharmacodynamics of the component (+)-(*R*)- and (−)-(*S*)-thalidomide enantiomers (hereafter referred to as R- and S-thalidomide); moreover, these are biotransformed to a number of potentially active chiral and achiral metabolites [5,6]. Many methods have been developed to quantify thalidomide and its metabolites from a variety of biological matrices [7–17]. Most of these methods are achiral, being based on HPLC using reverse phase separation with UV, MS (mass spectrometry) or tandem MS detection. However, increasing recognition of the potential importance of stereoselectivity in thalidomide 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

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electrophoresis (CE) or electrochromatography (CEC) technology [18–33]. Vancomycin, a macrocyclic antibiotic, has been utilized as a CSP for separating a number of racemic drugs [34], including thalidomide [35]; however, there has yet been no reported use of a vancomycin-CSP for quantitative analysis of thalidomide concentrations in biological samples.

As part of a program to gain a better understanding of thalidomide pharmacokinetics in a laboratory rodent model of GBM, a quantitative chiral HPLC method was required to determine its enantiomers in serum, tumours and tissues of animals treated with thalidomide and combinations of thalidomide and chemotherapy, as well as in serum of patients undergoing thalidomide treatment. As no published methods were available for the routine and high throughput determination of thalidomide enantiomers in relevant biological samples, we developed and validated this relatively simple method of liquid/liquid extraction of thalidomide, with vancomycin HPLC-CSP for enantiomer separation, and UV detector quantitation. The method reported here has now been successfully applied to serum in our ongoing pharmacokinetic studies in human patients undergoing thalidomide treatment for mesothelioma, and to serum and tissue samples from a laboratory rodent model using transplanted 9 l gliosarcoma.

2. Experimental

2.1. Overview

The stability of thalidomide in aqueous media is the most important issue to assure the reliability of the assay. Samples need to be promptly cooled, acidified and stored at −20° in order to avoid hydrolytic degradation of thalidomide [5–14]. The method developed was simple, with good precision and accuracy. Serum samples, buffered to pH < 4 with 1:1 volume of citrate-phosphate buffer (pH 2, 0.2 M), stored in −80 °C, were tested over 1 year and no analyte degradation was observed. Chiral separation on a vancomycin-CSP was easily achieved using a mobile phase of 14% acetonitrile in 20 mM ammonium formate adjusted to pH 5.4. The respective biological studies were approved by the relevant ethics review panels.

2.2. Reagents

Thalidomide (racemate), R-thalidomide, S-thalidomide and phenacetin (internal standard) were obtained from Sigma–Aldrich (Sydney, Australia). OmniSolv grade acetonitrile, HiPerSolv methanol, AnalaR grade diethyl ether, ammonium formate, formic acid and citric acid, Univar grade sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate were obtained from Crown Scientific (Sydney, Australia). Water was purified by a Milli-Q water system obtained from Millipore (Sydney, Australia).

2.3. Instrumentation and chromatography

Analyses were performed on a Hewlett Packard 1100 Series High Performance Liquid Chromatograph equipped with an auto-sampler and photodiode array detector and a vancomycin column (Chirobiotic V^{TM}, 250 mm × 4 mm ID; Astec-Advanced Separation Technologies Inc., Whippany, USA, purchased from Alltech, Sydney, Australia) with a guard column of the same type (20 mm × 4 mm ID) or of C18 (SecurityGuard^{TM}, 4 mm × 2 mm ID; Phenomenex, Australia). The mobile phase consisted of 14% acetonitrile in 20 mM ammonium formate, adjusted to pH 5.4 with formic acid; the flow rate was 1.0 ml/min. The UV wavelength of detection was 220 nm. A Medos JAK evaporator (Dynavac Engineering, Sydney, Australia) was used for evaporation of solvent after the extraction procedure at 40 °C, under vacuum.

2.4. Sample preparation

2.4.1. Rat samples

In the rodent model, adult female Fischer rats were implanted with 9 l gliosarcoma tumours either intracranially, subcutaneously or both, for investigations of the effectiveness of thalidomide with and without standard chemotherapy as treatment for GBM. Brain, brain tumour, subcutaneous tumour and blood samples were collected under general anaesthesia at the end of the treatment. Blood, collected by cardiac puncture into polypropylene tubes (5 ml), was centrifuged at 10 °C; serum was harvested and mixed (1:1, v/v) with citrate-phosphate buffer (0.2 M, pH 2) to stabilize thalidomide, and stored at −80 °C until analysis. A preliminary experiment with thalidomide spiked serum mixed (10:1, v/v) with citrate-phosphate buffer (0.1 M, pH 2) and stored at −80 °C for 4 weeks gave evidence that net S- to R-enantiomerization (of approximately 10%) could be occurring during storage and/or thawing; hence, 0.2 M buffer mixed (1:1, v/v) with serum was adopted for the systematic studies. Tissue samples were immediately frozen, then homogenised (1:4, w/v) in citrate-phosphate buffer (0.2 M, pH 2), and also stored at −80 °C until analysis.

2.4.2. Human samples

Blood samples were collected from patients being treated with thalidomide for treatment of mesothelioma as part of a clinical trial. The samples were collected and processed using the final method as for the rat samples.

2.5. Standards

A stock solution of thalidomide (2 mg/ml) was prepared in 50% methanol in acetonitrile. Working standard solutions were diluted with 50% methanol in citrate-phosphate buffer (pH 2, 20 mM). Calibration standards were made by adding volumes of thalidomide working solutions to drug-free serum or to tissue homogenate, buffered with a 1:1 volume of citrate-phosphate buffer (pH 2, 0.2 M), to give the final thalidomide concentrations in the range of 0.1–20 μg/ml. At least six concentrations were used to form the calibration curve in the assay. The working internal standard solution (phenacetin, 0.28 mg/ml) was prepared in 50% methanol in citrate-phosphate buffer (pH 2, 20 mM).

2.6. Extraction

Serum or blood samples (0.2 ml) including calibration standards (up to 0.6 ml) were pipetted into Eppendorf tubes (1.5 or 2 ml) containing internal standard (phenacetin, 50 μl, 0.28 mg/ml). Diethyl ether (1.1 ml) was added and the tubes capped. The mixture was shaken for 1 min, centrifuged at 1500 × *g* (5 min), then frozen in dry ice (∼15 min). The organic layer was decanted into another set of Eppendorf tubes and evaporated to dryness under vacuum (40 °C). The extract was reconstituted in 100 μl 50% methanol in citrate-phosphate buffer (pH 2, 20 mM), vortex mixed (10 s), sonicated (1 min), centrifuged (1 min), then transferred into a 250 μl polypropylene insert. An aliquot (5–20 μl) was injected onto the HPLC column.

2.7. Method validation

2.7.1. Accuracy and precision

Accuracy (deviation from standards, *D*%) and within-assay precision (coefficient of variation, CV%) was determined by six replicates of serum samples containing 0.1, 1, 5 and 10 μg/ml thalidomide. Between-assay precision (coefficient of variation, CV%) was determined from the results of six assays of 0.1, 1.0, 5.0 and 20 μg/ml *rac*-thalidomide spiked calibration standards samples.

2.7.2. Recovery and assay limits

Recovery of the analytes after extraction (expressed as percentages) was determined by comparing peak areas from extracted spiked serum samples with those from standard solutions directly injected. Limit of detection was determined from the least concentration giving an S/N level >3, being a peak distinguishable from baseline noise. The lower limit of quantitation (LOQ) was determined from the lowest concentration measurable with the given precision and accuracy <20%.

2.7.3. Stability

In a preliminary study, six rat serum samples from pharmacotherapy studies were mixed (1:1) with citrate-phosphate buffer (0.1 M, pH 2) as a stabilizer, stored at −80 °C, and assayed initially, again after 2, 6 and 12 months storage. In the systematic study, stability was determined by preparing samples at low, medium and high concentrations (respectively, 0.5, 5.0 and 20 μg/ml for rat serum and 0.2, 2.0 and 10 μg/ml for human serum). These samples were assayed at time intervals of 3 days, 1 week, 2 weeks, 1 month, 2 months, 6 months and 1 year, and by three freeze–thaw cycles in 3 days. The samples were prepared as follows: the bulk blank serum was spiked to the required concentrations with thalidomide and diluted (1:1, v/v) with citrate-phosphate buffer (0.2 M, pH 2). The spiked serum at each concentration was divided into three sets of 5 ml blood collecting tubes and stored at −80 °C until analysis. Triplicate samples from the three concentration sets were assayed after each time of storage.

3. Results

3.1. Chromatography

The respective retention times for R-thalidomide, S-thalidomide and internal standard phenacetin were 8.6, 15.0 and 7.2 min with the preferred mobile phase consisting of acetonitrile (14%) in ammonium formate (20 mM) adjusted to pH 5.4. Typical chromatograms from extracted samples are shown in Fig. 1 from which it is clear that the vancomycin-CSP completely resolved the thalidomide enantiomers. Separation of the enantiomers could be achieved with 12–35% acetonitrile in the mobile phase. However, the preferred mobile phase was selected (and micro-adjusted as required) on the basis of several criteria: giving a clear separation of R-thalidomide from phenacetin, avoidance of an endogenous peak sometimes occurring in the serum samples between R-thalidomide and phenacetin, providing a shorter run time and a stable baseline. The mobile phase had an optimal pH of pH ∼5.4. Thalidomide undergoes hydrolytic degradation at higher pH, whereas a lower pH tends to produce a poorer baseline, noticeably around the times of the phenacetin and R-thalidomide peaks, due to interference from citrate carried over from the buffer.

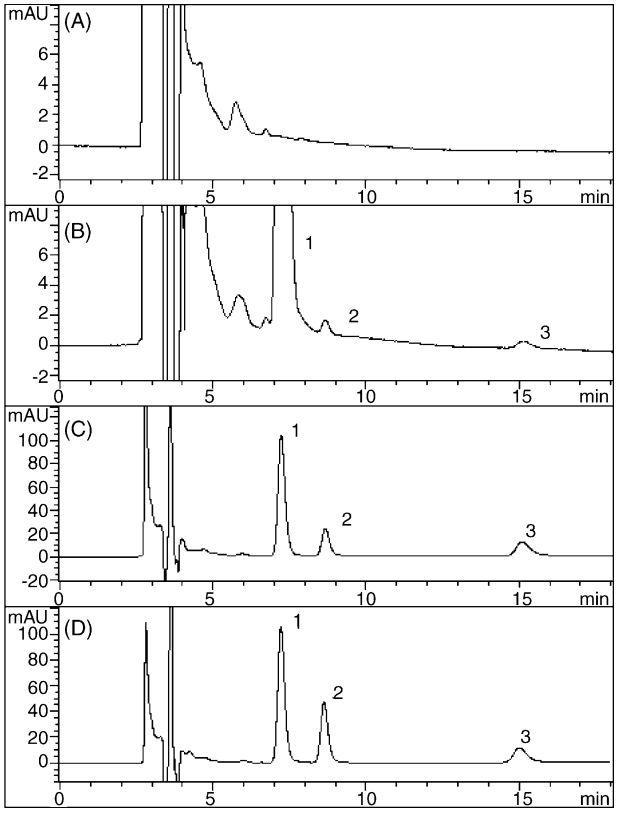

Fig. 1. Chromatograms showing (A) blank rat serum (0.2 ml) extract; (B) the separation of phenacetin (internal standard) [1], R-thalidomide [2] and S-thalidomide [3] from rat serum (0.2 ml) spiked at the lower limit of the quantitation; (C) spiked rat serum (0.2 ml) containing 5.0 μg/ml thalidomide (0.6 ml serum extracted); (D) a sample of rat serum (0.2 ml) found to be containing 5.1 μg/ml R-thalidomide and 2.5 μg/ml S-thalidomide.

Potential interference from a number of drugs often used in anaesthesia and pain management of human and experimental animal patients was tested. It was found that the analytes could be separated from the intravenous anaesthetics ketamine, thiopentone and pentobarbitone, the local anaesthetics lignocaine and ropivacaine, and the analgesics paracetamol, ketorolac, flurbiprofen, diclofenac and tolmetin, but ketoprofen and naproxen were not adequately separated from the phenacetin internal standard with this system.

3.2. Method validation

The calibration standard curves for most of the assays had coefficient of determination (r^2) > 0.999. Linearity ranged from 0.1 to 20 μg/ml thalidomide concentrations in samples, i.e. 0.05–10 μg/ml of each enantiomer. The accuracy, intra-assay precision and recovery for rat and human serum samples, rat brain and subcutaneous tumour tissue samples are summarised in Table 1.

Overall, the assays demonstrated accuracy (*D*%) and precision (CV%) were each <5%. The LOQs for rat and human serum were, respectively, ~0.025 μg/ml for each enantiomer, with both *D*% and CV% being <20%. The extraction recovery varied from a lowest value of 54% (with 0.6 ml human serum containing 10 μg/ml S-thalidomide) to 81% (with 0.2 ml rat serum containing 10 μg/ml R-thalidomide). Comparisons of analyses of human serum spiked with 10 μg/ml *rac*-thalidomide in 0.6 ml, 20 μg/ml in 0.4 ml and 20 μg/ml in 0.6 ml, gave respective mean recoveries of R- and S-thalidomide of 69 and 70%, 76 and 77% and 60 and 61%. Recovery from rat tissue matrices (volumes of 0.2 ml) ranged from 75 to 100%. Recovery of phenacetin (internal standard) averaged 85%, regardless of the volume of matrix extracted.

The inter-assay precision (Table 2) was derived from data obtained from a number of sequential serum assays (3–7 assays) over a year and consisted of 10–16 replicate samples. The CV% values were all <10% for concentrations of 0.05–10 μg/ml of both enantiomers.

3.3. Stability

Tables 3 and 4 summarise the data obtained from studying the stability of thalidomide in spiked rat and human serum over a 1 year period. The average concentrations and their variability were calculated to show the CV% over the year; these were all within 8% indicating no significant degradation of R-thalidomide or S-thalidomide within 1 year with concentration ratios of approximately 1.0 for both rat and human serum at all thalidomide spiked concentrations. The corresponding stability profiles (percentages of remaining thalidomide in the samples, compared to Day 1 assay, mean of triplicates) showed that the remaining thalidomide ranged from 96 to 117% for rat serum, and 97 to 115% for human serum, after 1 year. In addition, it was observed that aqueous thalidomide standards, buffered in the same way, stored at −80 °C for over 2 years, showed no change in concentration, and the enantiomeric ratio did not deviate from unity.

The preliminary study with the rat serum samples from thalidomide pharmacotherapy and stabilized with citrate-phosphate buffer (0.1 M, pH 2) also supplied useful pilot

Table 1
Method validation data for thalidomide (thal) in rat and human serum, rat brain and subcutaneous tumour tissue homogenate

Spiked concentrations thalidomide (μg/ml)	Observed concentrations (μg/ml), mean (n = 6)		Accuracy *D*%		Precision CV%		Recovery (%)	
	R-thal	S-thal	R-thal	S-thal	R-thal	S-thal	R-thal	S-thal
Rat serum (0.2 ml)								
0.1	0.048	0.048	−4	−4	2.8	3.2	72	73
0.5	0.23	0.23	−7	−8	0.7	1.2	71	70
5	2.43	2.41	−3	−4	0.6	0.5	75	74
20	10.04	10.05	0	1	1	0.8	81	80
Human serum (0.6 ml)								
0.05	0.026	0.026	4	2	3.4	5.1	63	54
0.1	0.052	0.044	4	−12	1.8	4.7	63	54
1	0.51	0.51	2	2	0.8	1	62	62
5	2.44	2.44	−2	−2	0.4	0.4	60	60
10	4.98	5.02	0	0	4.1	4.1	69	70
20	9.10	9.12	−9	−9	9.1	9.2	56	56
Rat brain homogenate (0.2 ml)								
0.1	0.064	0.063	28	26	3.9	5.5	95	97
0.5	0.28	0.28	10	11	5.4	6.7	84	87
5	2.47	2.49	−1	0	2.2	2.7	75	78
20	9.92	10.05	−1	1	1.6	1.6	75	78
Rat subcutaneous tumour homogenate (0.2 ml)								
0.1	0.061	0.066	22	31	1.6	3.6	93	100
0.5	0.26	0.26	2	3	2.3	0.7	79	79
5	2.44	2.45	−3	−2	1.5	1.5	75	75
20	10.05	10.04	1	0	0.9	1.0	80	80

Accuracy expressed as *D*%: the deviation of the nominal values; CV% (coefficient of determination) shown for intra-assay precision; recovery were the percentage of the extracted thalidomide.

Table 2
Inter-assay precision for thalidomide (thal) in rat and human serum

Spiked concentration thalidomide (μg/ml)	Observed concentrations (μg/ml), mean of *n* replicates		S.D.		Precision CV%		Number of replicates (*n*)	Number of assays
	R-thal	S-thal	R-thal	S-thal	R-thal	S-thal		
Rat serum								
0.1	0.048	0.047	0.005	0.004	9.5	8.4	10	3
0.5	0.23	0.23	0.016	0.018	6.7	7.7	14	7
1	0.48	0.49	0.015	0.015	3.2	3.0	14	7
5	2.42	2.43	0.08	0.08	3.4	3.5	14	7
20	10.02	10.03	0.25	0.26	2.5	2.6	14	7
Human serum								
0.1	0.054	0.050	0.005	0.003	8.8	6.7	14	7
1	0.51	0.50	0.009	0.013	1.7	2.6	14	7
5	2.47	2.47	0.04	0.04	1.6	1.8	14	7
10	5.02	5.02	0.09	0.10	1.9	2.0	14	7
20	9.29	9.31	0.72	0.72	7.8	7.7	16	6

data concerning chiral stability over a 1 year storage period. There was no significant difference in thalidomide concentration (repeated measures ANOVA $F_{5,3} = 1.63$, $P = 0.24$) (Fig. 2) or in enantiomeric excess ($F_{5,3} = 0.12$, $P = 0.94$) (Fig. 3) during this period.

4. Discussion

This paper reports the development, validation and application of a simple liquid/liquid extraction and HPLC-UV method for the routine separation and quantification of the enantiomers of thalidomide using a proprietary vancomycin-CSP column, and a simple mobile phase consisting of 14% acetonitrile in 20 mM ammonium formate adjusted to pH 5.4.

The main advantages of using a vancomycin stationary phase (as compared to a glycoprotein CSP column) are that it has stability from 0 to 100% of organic modifier, with similar analyte selectivity, and with a higher sample capacity. A combination of high resolution and high efficiency was demonstrated so that a wide range of the mobile phase composition could be tolerated to achieve complete enantiomeric separation. In our trials of mobile phase, we explored percentages of organic modifier of up to 35% acetonitrile, over a pH range from pH 3 to 7, complete enantiomeric enantiomers (Fig. 4), although a different

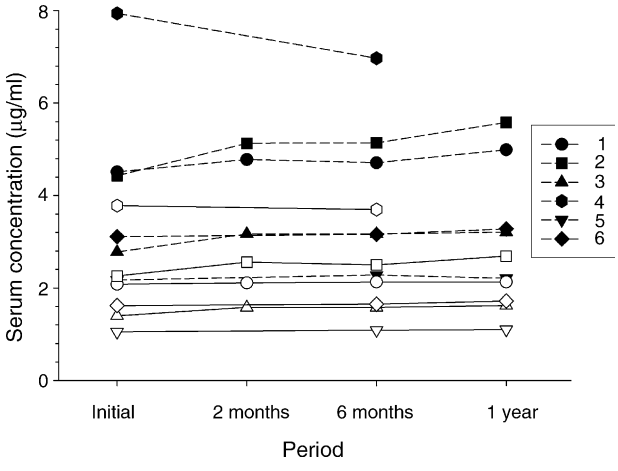

Fig. 2. Results from a preliminary study showing results of 1 year storage on the serum concentrations of R- and S-thalidomide (respectively paired closed and open symbols) obtained during oncologic pharmacotherapy in six rats; there was no significant difference of thalidomide concentration between periods over a wide concentration range ($P = 0.29$, repeated measures ANOVA).

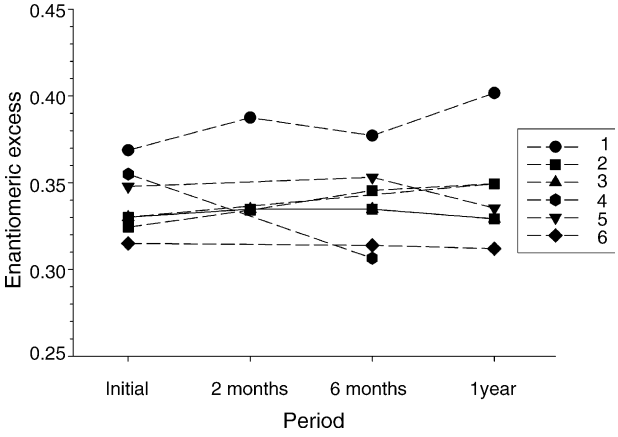

Fig. 3. Results from a preliminary study showing calculated enantiomeric excess as an index of the relative rates of enantiomerization performed on six rat serum samples shown in Fig. 2; there was no significant difference of enantiomeric excess between periods ($P = 0.94$, repeated measures ANOVA).

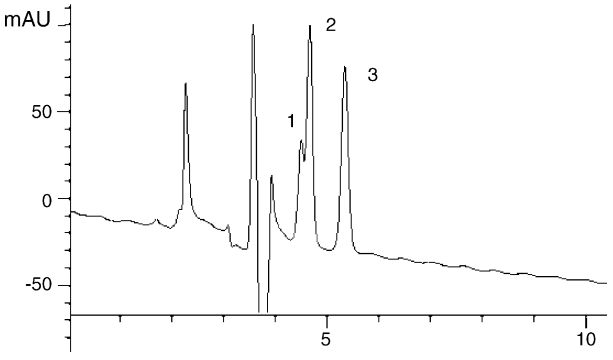

Fig. 4. Chromatogram showing resolution of R- [2] and S-thalidomide [3] (but with incomplete resolution of phenacetin, internal standard) [1], when using a mobile phase of 30% acetonitrile in ammonium formate (20 mM, pH 5.4).

Table 3
Thalidomide stability over 1 year: rat serum data

Time period	Observed concentrations (μg/ml), mean ($n = 3$)		CV%		Thalidomide remaining (%)		Enantiomer ratio (R)/(S)
	R-thal	S-thal	R-thal	S-thal	R-thal	S-thal	
Thalidomide level (racemate)							
Low: ∼0.5 μg/ml							
1 day	0.243	0.228	9.4	6.1	100	100	1.07
3 day	0.259	0.242	2.3	2.3	107	106	1.07
F–Thaw (3)	0.246	0.241	1.9	3.5	101	106	1.02
1 week	0.240	0.234	2.8	0.7	99	102	1.03
2 weeks	0.251	0.253	1.6	1.2	104	111	0.99
1 month	0.256	0.254	2.6	2.6	106	111	1.01
2 months	0.258	0.251	0.9	2.2	106	110	1.03
6 months	0.256	0.264	1.3	2.7	105	116	0.97
1 year	0.252	0.266	1.4	3.4	104	117	0.95
Average	0.251	0.248					1.00
S.D.	0.007	0.013					0.03
CV%	2.7%	5.2%					3.1%
Med: ∼5.0 μg/ml							
1 day	2.47	2.45	1.4	1.5	100	100	1.01
3 day	2.37	2.37	0.7	0.5	96	97	1.00
F–Thaw (3)	2.35	2.33	1.0	1.2	95	95	1.01
1 week	2.51	2.5	0.3	0.3	102	102	1.00
2 weeks	2.65	2.65	1.5	1.2	107	108	1.00
1 month	2.41	2.43	3.6	3.6	98	99	0.99
2 months	2.68	2.58	0.9	0.4	108	105	1.04
6 months	2.73	2.77	2.6	3.3	111	113	0.99
1 year	2.82	2.86	1.9	1.7	114	116	0.99
Average	2.55	2.55					1.00
S.D.	0.17	0.18					0.02
CV%	6.7%	7.1%					1.8%
High: ∼20 μg/ml							
1 day	8.91	8.91	0.6	0.5	100	100	1.00
3 days	8.56	8.58	0.7	0.7	96	96	1.00
F–Thaw (3)	8.45	8.53	0.3	0.2	95	96	0.99
1 week	9.17	9.19	1.1	1.1	103	103	1.00
2 weeks	9.04	9.09	5.1	5.2	101	102	0.99
1 month	9.03	9.15	1.0	1.1	101	103	0.99
2 months	9.87	9.93	0.8	0.9	111	111	0.99
6 months	9.92	9.94	1.3	1.2	111	112	1.00
1 year	10.3	10.3	1.6	1.6	115	116	1.00
Average	9.25	9.29					0.99
SD	0.64	0.63					0.01
CV%	6.9%	6.8%					0.5%

F–Thaw (3): freeze–thaw three cycles.

internal standard may be required. With phenacetin as the internal standard, and with an endogenous peak being found in some samples between phenacetin and R-thalidomide, it was necessary to adjust the acetonitrile percentage between 14 and 25%; this adjustment was also appropriate to compensate for aging of the column before column rejuvenation. It was noted that when the pH of the mobile phase was lower than pH 4, the retention of citrate, carried over from the buffer, produced a poor baseline. In addition, thalidomide may become chemically unstable with pH > 6; we chose pH 5.4 for the mobile phase, and found this appropriate for the assay. Initially, we used a guard column of the same kind to protect the main column; however, we found that a C18 guard column worked as well without affecting separation of the analytes and was less expensive.

We adapted published methods of sample preparation, internal standard selection and extraction solvent [11,13–15] in the following ways. Eriksson et al. [13] suggested storage of samples at −20 °C and to analyse samples within 75 days. We needed longer storage time because of the study protocols; we found that the suggested buffer concentration of 0.025 M was not enough to maintain the serum at pH < 6 after 4 weeks at −80 °C. Enantioselective degradation was found by a 10% excess concentration of R-thalidomide after 4 weeks. In our method, samples were buffered with a 1:1 volume of citrate buffer (pH 2, 0.2 M) to reduce the serum to pH < 4. We extended our stability study from 6 months to 1 year. The results indicated that by adding equal volumes of citrate-phosphate buffer (pH 2, 0.2 M) to serum, the thalidomide remained stable for at

Table 4
Thalidomide stability over 1 year: human serum data

Time period	Observed concentrations (μg/ml), mean ($n = 3$)		CV%		Thalidomide remaining (%)		Enantiomer ratio (R)/(S)
	R-thal	S-thal	R-thal	S-thal	R-thal	S-thal	
Thalidomide level (racemate)							
Low: ∼ 0.2 μg/ml							
Day 1	0.066	0.065	1.1	5.8	100	100	1.02
Day 3	0.065	0.064	3.1	3.4	98	98	1.02
F–Thaw (3)	0.069	0.068	5.0	6.3	104	104	1.01
1 week	0.069	0.068	1.9	1.2	104	104	1.01
2 months	0.069	0.064	3.9	8.2	104	98	1.08
6 months	0.073	0.068	3.8	6.7	110	105	1.07
Average	0.069	0.066					1.04
S.D.	0.003	0.002					0.03
CV%	4.1%	3.1%					3.0%
Day 1 (2#)	0.10	0.10	1.1	2.9	100	100	1.05
1 year (2#)	0.11	0.11	2.4	1.4	110	113	1.02
Med: ∼2.0 μg/ml							
Day 1	0.67	0.67	1.5	0.8	100	100	1.00
Day 3	0.67	0.66	1.4	1.6	99	99	1.00
F–Thaw (3)	0.65	0.67	1.1	1.5	97	100	0.98
1 week	0.70	0.70	1.2	1.4	105	104	1.00
2 months	0.73	0.72	1.1	1.1	109	107	1.01
6 months	0.77	0.74	2	2.1	115	111	1.04
Average	0.70	0.69					1.01
S.D.	0.04	0.03					0.02
CV%	6.4%	4.6%					2.1%
Day 1 (2#)	1.07	1.06	1.1	1	100	100	1.01
1 year (2#)	1.14	1.14	0.2	0.6	107	108	1.00
High: ∼10 μg/ml							
Day 1	3.44	3.46	3.1	2.9	100	100	1.00
Day 3	3.43	3.43	0.6	1	100	99	1.00
F–Thaw (3)	3.44	3.45	1.1	1.4	100	100	1.00
1 week	3.67	3.68	1.2	1.3	107	106	1.00
2 months	3.76	3.76	0.4	0.3	109	109	1.00
6 months	3.95	3.91	0.5	1.1	115	113	1.01
Average	3.62	3.61					1.00
S.D.	0.21	0.20					0.01
CV%	5.9%	5.5%					0.5%
Day 1 (2#)	4.84	4.84	0.6	0.8	100	100	1.00
1 year (2#)	5.45	5.47	1.4	1.4	113	113	1.00

least 1 year. Moreover, we used a low pH buffer (50% methanol in citrate-phosphate buffer (pH 2, 20 mM) in the thalidomide working standards and in the reconstitution of the final extract of the samples. This assured the stability of thalidomide during sample work-up (3–5 h at room temperature) and subsequent HPLC runs. When a large batch of samples was assayed, the total run time could be up to 24 h. We found that the results from re-running samples that had been sitting in sample tray for 24 h were not different to those when first run (data not shown).

In addition, Lyon et al. [14] used a ratio of 0.2 ml serum to 5 ml diethyl ether. We used higher ratio to achieve greater sensitivity, consistent with the needs of the application in rats. Others have recently demonstrated similar sensitivity for studies in rats using a serum protein precipitation and achiral technique [17]. In our general method, we scaled down the extraction volumes: 0.2 ml serum was extracted with 1.1 ml diethyl ether in a 2 ml tube, then transferred organic layer to another 1.5 ml tube, dried, reconstituted in 0.1 ml methanolic buffer, then injected 5–20 μl onto the column, depending on the expected thalidomide concentration. Accordingly, the extraction efficiency was acceptable with a recovery of 50–70%, and with less solvent consumption. For the human serum assay, 0.6 ml serum was extracted (in a 2 ml tube) to increase the sensitivity. It was found that the extracted precision for 0.6 ml serum spiked with 20 μg/ml thalidomide had a relatively higher CV% and lower recovery than at 10 μg/ml, or 0.4 ml spiked with 20 μg/ml. This indicated that 0.6 ml extraction of the spiked 20 μg/ml sample was too much for the small extraction volume. The linearity range was only up to 10 μg/ml for 0.6 ml serum extraction or 20 μg/ml for

0.4 ml serum extraction. The larger volume of (human) serum, however, also produced a relatively poorer recovery; this was influenced, no doubt, by the small (2 ml) tube used for extraction, as well as by the relatively non-polar solvent (diethyl ether) used. A greater recovery of thalidomide could have been produced with a more polar (water immiscible) solvent, but with greater likelihood of co-extracting more polar substances, including thalidomide hydroxylated and carboxylated metabolites [4–6,18,36–38], and endogenous substances. As the precision and reproducibility were both acceptable, the recovery was considered adequate. These, more polar, metabolites were not tested in the system; they would have been expected to elute more rapidly than thalidomide, consistent with their greater polarity. The hydroxylated metabolites are reported to be minor to negligible in humans [36–38].

The issue of thalidomide enantiomerization merits further comment. The preliminary study on serum from rat pharmacotherapy studies found both chemical and chiral stability during storage for 12 months. Hence, if enantiomerization was occurring, then it must have been occurring at bi-directional rates that were insignificantly different, and thus not detectible. Other analyses suggest similar rates of R- to S- and S- to R-enantiomerization, albeit under very different environmental conditions, i.e. at or near physiological pH, at or near body temperature [24,27,29,39]. However, it is interesting to note that inversion of both enantiomers was insignificant at pH 2.03 and 3.98 [39]. It is clear that any future study of thalidomide enantiomerization during storage should incorporate proper conditions, i.e. low pH, deeply frozen temperatures, in appropriate biological media from relevant species.

5. Conclusion

The vancomycin HPLC-CSP column is a good choice for separation and quantification of thalidomide enantiomers. Thalidomide in rat samples and human serum samples, buffered to pH < 4 with equal volume of citrate-phosphate buffer (pH 2, 0.2 M), was found to be chemically and chirally stable for at least 1 year stored at −80 °C. The validated method reported here has been successfully applied to our pharmacokinetic studies in human patients undergoing thalidomide treatment for mesothelioma, and to serum and tissue samples from a laboratory rodent model using transplanted 9 l gliosarcoma. The assay was easy to perform and allowed a high throughput. An example of the applied method demonstrating enantioselectivity in the pharmacokinetics of thalidomide in rats is given in Fig. 5, thus reinforcing the need for considering the relevance of chirality in thalidomide pharmacology.

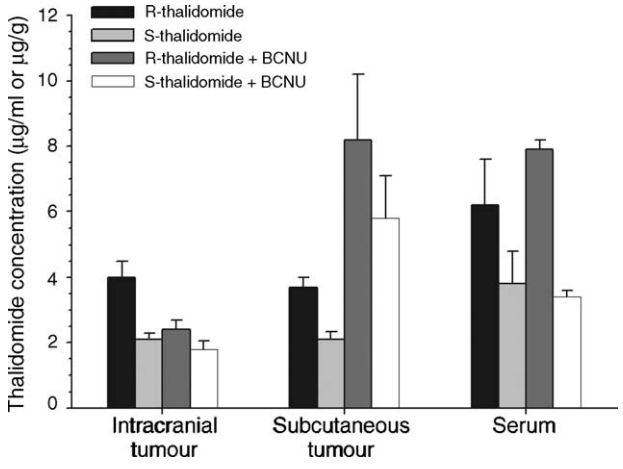

Fig. 5. An example of the described method applied to serum and tissue samples from a laboratory rodent model using transplanted 9 l gliosarcoma and treatment with thalidomide alone, and together with BCNU [1,3-*bis*(2-chloroethyl)-1-nitrosourea]. A set of data (mean and S.E.M., $n = 8$) from rat serum and tumour experiments is shown.

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