Quantitative determination of thalidomide in human serum with high-performance liquid chromatography using protein precipitation with trichloroacetic acid and ultraviolet detection

Javier Sastre Toraño[a], Annelies Verbon[b], Henk-Jan Guchelaar[a],*

[a]Pharmaceutical and Toxicological Laboratory, Department of Pharmacy, E0127, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands
[b]Department of Infectious Diseases, Tropical Medicine and AIDS, E0127, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

Abstract

A validated and precise reversed-phase high-performance liquid chromatographic method for the determination of thalidomide in serum, with phenacetin as an internal standard, is described. Protein precipitation, using trichloroacetic acid, was used for clean-up. The aliquot was chromatographed on a octadecyl column, using an eluent composed of 250 ml 0.01 M potassium dihydrogenphosphate, adjusted to a pH of 3.0 with a 43% phosphoric acid solution, mixed with 750 ml methanol. Ultraviolet detection was used at an operation wavelength of 220 nm. Hydrolytic degradation was prevented during analysis by acidification of samples with the precipitation reagent. Thalidomide and phenacetin were found to have retention times of 7.9 and 15.0 min, respectively. Recoveries ranging from 79 to 84% were found for both components, with reproducibility relative standard deviations of 0.8–3% and repeatability coefficients of 1.2–3%. A mean correlation coefficient of 0.9995 was found for the linear calibration curve ($n=2$) of thalidomide with limits of quantitation of 0.222–21 mg/l. The method appeared to be feasible for pharmacokinetic studies with thalidomide. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

In the recent years thalidomide (2-phtalimido-glutaramide) (Fig. 1) has been shown to be a valuable drug for a variety of illnesses. It has been used for decades in erythema nodosum leprorum with good result. More recently, thalidomide has been shown to be effective in major apthous ulcers, Bechet's syndrome, graft-versus-host disease and in infectious diseases such as mycobacterial infections and HIV. The exact mechanism is yet unknown but is hypothesized to include a decrease in tumor necrosis factor (TNF)-α levels, inhibition of interleukin-12 production and co-stimulation of CD8 lymphocytes. Since the association between serum levels and effect is not clear, the first step towards elucidation of the immunosuppressive mechanism would be an easy method to determine thalidomide levels in serum.

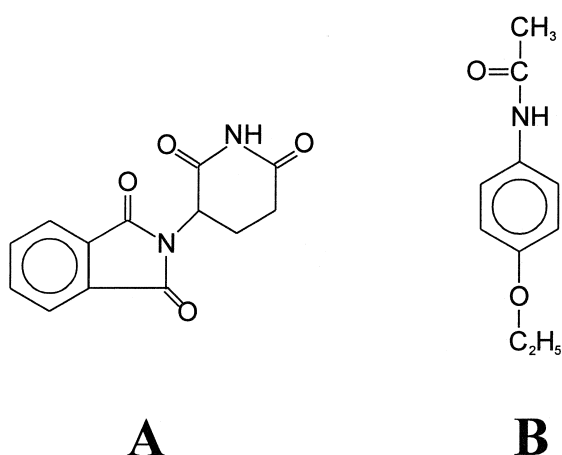*Corresponding author. Tel.: +31-20-5663-474; fax: +31-20-6972-291.

Fig. 1. Chemical structures of thalidomide (A) and phenacetin (B).

Thalidomide is known to degrade rapidly in solutions at both physiological pH and alkaline conditions. The chromatographic assays thus far described [1–5] have the disadvantage of a time consuming sample pretreatment, such as liquid–liquid and solid-phase extraction procedures. In addition, some of these methods use an ion-pair reagent to analyze hydrophylic degradation products or gradient chromatography, which increases the processing time and therefore the risk of degradation of thalidomide in the samples during analysis. Alternative methods for the analysis of thalidomide describe the use of acidification of serum samples to stabilize thalidomide during storage and clean-up. Additionally, as thalidomide is a chiral compound, separation of the $(+)$-(R)- and $(-)$-(S)-enantiomers of thalidomide has been reported [6].

We describe a fast, practical and precise method for analysis of thalidomide in human serum, with phenacetin (Fig. 1) as internal standard, using protein precipitation with trichloroacetic acid. This method proved to be reliable without any signs of hydrolytic degradation of thalidomide during the procedure.

2. Experimental

2.1. Chemicals and reagents

Thalidomide was obtained from Duchefa (Haar-

lem, The Netherlands), phenacetin (purity>99%) was obtained from Bufa (Uitgeest, The Netherlands). Sterile distilled water was from Braun NPBI (Emmer-Compascuum, The Netherlands). Methanol (gradient grade), phosphoric acid (analytical-reagent grade), trichloroacetic acid, 20% (analytical-reagent grade) and potassium dihydrogenphosphate (analytical-reagent grade) were purchased from Merck (Amsterdam, The Netherlands). All thalidomide stock solutions were freshly prepared in methanol while phenacetin stock solutions were stored in methanol at 3–8°C.

2.2. Chromatography

The mobile phase was prepared by mixing 750 ml methanol with 250 ml of a solution which was composed of potassium dihydrogenphosphate (0.01 mol/l water) adjusted to a pH of 3.0 with a 43% phosphoric acid solution. The solvent was filtered and degassed through a 0.22-μm filter from Millipore (Etten Leur, The Netherlands).

The chromatography system consisted of: a Rheodyne 7125 injector, a Waters M515 pump at a flow-rate of 1.5 ml/min, a Waters 996 diode array detector at an operation wavelength of 220 nm and a Millennium^{32} (version 3.05) chromatographic data system from Waters (Etten-Leur, The Netherlands).

Separation was performed on a 125×4.6 mm Discovery column packed with 5 μm C_{18} particles with a 20×4.6 mm Discovery C_{18} guard column from Supelco (Zwijndrecht, The Netherlands).

2.3. Sample preparation

In a 10-ml disposable glass tube, 20.0 μl of a 0.25 mg/ml phenacetin internal standard solution was added to a 0.50 ml serum sample, containing thalidomide. A 500-μl volume of a 10% trichloroacetic acid solution was added under constant vortex mixing, to prevent local hyperconcentration, for 30 s. The solution was centrifuged for 5 min at 2700 *g*. A 20-μl volume of the aliquot was injected into the chromatographic system.

2.4. Validation of the thalidomide assay

2.4.1. Specificity and selectivity
For the examination on the presence of interfering

endogenous components, human serum from six different drug-free volunteers was tested. These samples were pretreated according to the sample preparation procedure, apart from the addition of internal standard solution. A reference solution containing thalidomide and phenacetin in serum was prepared and compared with the blank solutions.

Furthermore, several in this group of patients commonly used drugs, and these were tested for interference with the assay. These co-medications, tested at toxic concentrations, are listed in Table 1.

2.4.2. Recovery

Three serum standards with concentrations ranging over the limits of quantitation of the thalidomide assay, were determined in five-fold and compared with unpretreated reference solutions in eluent, prepared at similar concentrations as the standards.

For the determination of the recovery of thalidomide from human serum, three standards of thalidomide with concentrations extended over the therapeutic range were assayed in quintuple and compared with reference solutions prepared in eluent with similar concentrations as the pretreated solutions. The concentrations tested were 0.222, 4.44 and 11.1 mg/l.

The recovery of the internal standard phenacetin was determined in a similar way at the nominal concentration (10 mg/l), half the nominal concentration and twice the nominal concentration.

2.4.3. Precision

2.4.3.1. Repeatability. The samples used for the determination of the recovery were re-analyzed under varying conditions, such as the use of another chromatographic system with same characteristics and on consecutive days. The two data sets were individually used to calculate the within-run precision (intra-day variation) on both days.

The repeatability is defined as:

$$\text{repeatability RSD} = \frac{\sqrt{\text{MSwg}}}{\text{mean}} \cdot 100\%$$

where MSwg represents the mean square within both groups and RSD the relative standard deviation.

The mean square within groups was determined by the analysis of variance (ANOVA) test, performed with the statistical software program SPSS (version 6.1.3, SPSS).

In order to assure repeatable quantitative conditions, the repeatability RSD should not exceed the 15% limit.

Table 1
Retention times of co-medication tested on the interference with the assay

Co-medication	Tested quantity (µg)	Retention time (min)
Clofazimine	4	8.3
Cyclosporine	2.5	n.d.[a]
Dapsone	200	3.5
Desacethylrifabutin	0.2	n.d.
Desacethylrifampicin	0.2	1.4
Indinavir	60	4.0
Isoniazid	2	2.1
Lamivudine	100	2.2
Nevirapine	33	15.0
Rifabutin	0.2	n.d.
Rifampicin	0.2	2.5
Saquinavir	3	2.5
Stavudine	5	2.1
Sulfamemethoxazole	0.1	n.d.
Trimetoprim	0.02	4.8
Prednisolone	0.03	n.d.
Ritonavir	8	2.2
Zidovudine	80	4.0

[a] n.d. = Not detectable.

2.4.3.2. Reproducibility. The concentrations found in the samples determined on consecutive days were used to calculate the between-run variation (inter-day variation). The variation between the two individual sets of results obtained was determined, submitting the results to the ANOVA test; the mean square within groups and the mean square between groups were calculated. The reproducibility is defined as:

$$\text{reproducibility RSD} = \frac{\sqrt{\dfrac{\text{MSbg} - \text{MSwg}}{n}}}{\text{mean}} \cdot 100\%$$

where MSwg represents the mean square within groups, MSbg the mean square between groups and n the number of analysis of the sample quantified in one run.

In order to perform the assay with reliable reproducibility, the reproducibility RSD should be below 15%.

2.4.4. Limit of quantitation

The lower limit of quantitation (LLQ) is defined as the concentration which can be determined with a given precision. The LLQ is appointed at the concentration equal to $S/N = 5$. The thalidomide peak resembling this concentration should be well distinguishable from noise peaks.

The higher limit of quantitation (HLQ) is defined as twice the highest concentration in human samples to be expected.

Therapeutic values for thalidomide were reported to be 5–8 mg/l for the graft-versus-host disease [7,8], although other papers were reported with 0.5–1.5 mg/l serum concentrations to show therapeutic response [9]. For the verification of LLQ, the peak height in a chromatogram from a pretreated 0.2 mg/l thalidomide serum sample was compared with the noise signal.

2.4.5. Linearity

The linearity of the assay is the property of having a linear relationship between the thalidomide concentration and the detector response of the method ranging from LLQ to HLQ.

Five standards with concentrations between the limits of quantitation were assayed in twice fold. The results were submitted to the Student *t*-test using the statistical program STATCAL (STATCAL 6.50, University of Amsterdam, The Netherlands).

This program calculates the probability of the calibration curve's order performing the *t*-test to polynoma ($y = a + bx + cx^2 + \ldots.$) with different degrees. For a linear relationship, no significance ($P < 0.05$) should be found for orders surpassing the first degree. Furthermore the correlation coefficients of the curves should be 0.995 as minimal.

A calibration curve containing standards of 0.222 mg/l, 0.444 mg/l, 1.111 mg/l, 4.44 mg/l, 11.1 mg/l and 22.2 mg/l thalidomide in serum and 2.62 mg/l phenacetin as internal standard were used for the determination of the linearity of the thalidomide calibration curve.

2.4.6. Stability

Stock solutions of thalidomide and phenacetin in methanol (0.5 mg/ml) were stored at room temperature and 3–8°C. At appropriate time intervals a 5-µl volume of the stock solution was injected into the chromatographic system. The peak heights in the chromatograms obtained were quantitatively compared with peak heights from freshly prepared stock solutions in methanol.

Serum solutions spiked with thalidomide at a concentration of 4 mg/l were stored at room temperature, at 3–8°C and at −20°C and assayed, according to the sample preparation procedure, at appropriate time intervals. In order to assay the stability in human serum, the peak heights in the chromatogram obtained were quantitatively compared with the peak heights from freshly prepared in human serum.

2.5. Serum concentration curve of thalidomide in a human volunteer

A healthy 35-year-old male volunteer with a normal kidney and liver function took 400 mg of thalidomide orally at $t = 0$, 1 h after breakfast. Thalidomide serum concentrations were determined before and 1.5, 6, 6, 7.5, 24, 31 and 48 h after taking thalidomide.

3. Results

3.1. Validation of the thalidomide assay

3.1.1. Specificity and selectivity

Thalidomide and phenacetin chromatograph separately from each other and from endogenous components with retention times of 7.9 and 15.0 min, respectively. A representative chromatogram of blank serum spiked with thalidomide and phenacetin and a chromatogram of blank serum is shown in Fig. 2.

None of the tested co-medications interfered with the assay (Table 1). Nevirapin and clofazimin could interfere with the assay at extremely high toxic concentrations, resulting in partial overlap with phenacetin and thalidomide peak, respectively.

3.1.2. Recovery

Recoveries ranging from 78 to 81% were found for thalidomide. Phenacetin showed recoveries of 81 to 84%. Both drugs recoveries did not appear to be dependent upon the tested concentrations (Table 2).

3.1.3. Precision

3.1.3.1. Repeatability.
The repeatability of thalidomide ranged from 1.6 to 3% and for phenacetin from 1.2 to 3% (Table 2). The RSDs were all well below the 15% limit.

3.1.3.2. Reproducibility.
The samples assayed were found to have reproducibility RSDs well below the 15% limit (Table 2).
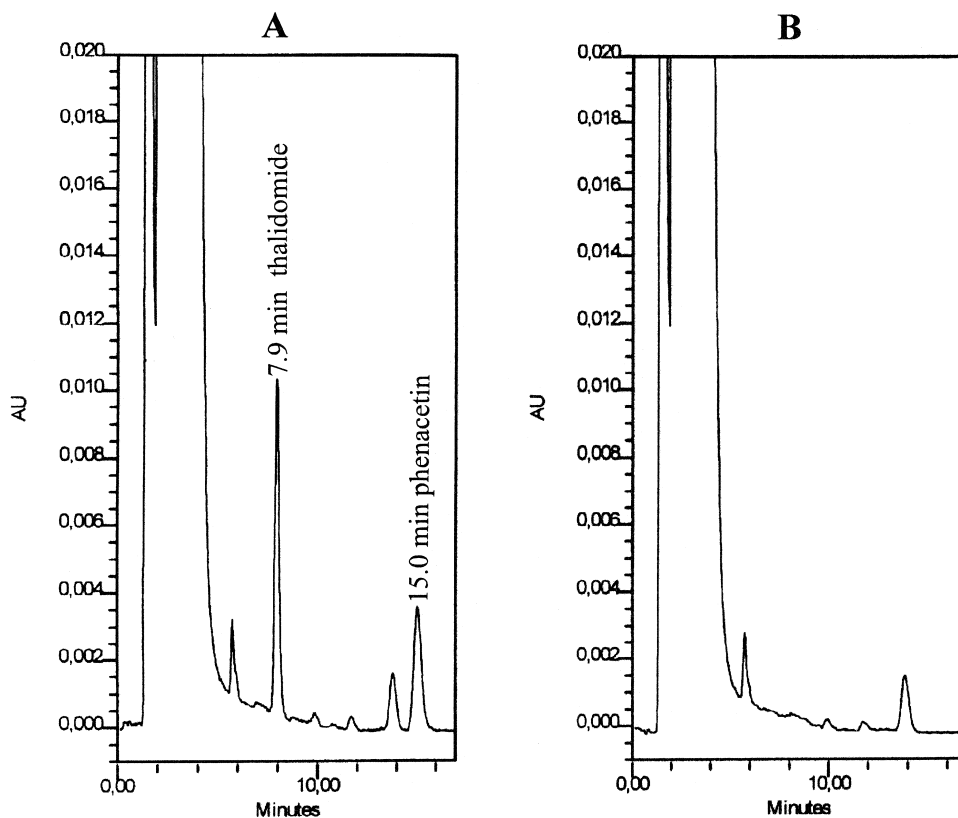

Fig. 2. Chromatograms of a serum aliquot (A) containing 5 mg/l thalidomide and phenacetin and a blank serum aliquot (B).

Table 2
Recoveries of thalidomide ($n=5$) and phenacetin ($n=5$) from serum samples, repeatability ($n=5$, runs=2) and reproducibility ($n=5$, runs=2) results

Compound	Concentration (mg/l)	Recovery (%)	Repeatability RSD (%)	Reproducibility RSD (%)
Thalidomide	0.222	79	3	5
	4.44	78	1.6	1.9
	11.1	81	2	0.8
Phenacetin	5.24	84	2	4
	10.5	82	3	1.8
	21.0	80	1.2	1.8

3.1.4. Limit of quantitation

The lower limit of quantitation of thalidomide was calculated at 0.222 mg/l. The chromatogram peak resembling this concentration was well distinguishable from noise peaks ($S/N \approx 5$). The higher limit of quantitation was estimated at 21 mg/l.

3.1.5. Linearity

Best curve fitting was obtained with first-degree regression, when applying the Student t-test to calibration points ranged between the limits of quantitation. The calibration curve of thalidomide was found to have a mean linear correlation coefficient of 0.9995 ($n=2$).

3.1.6. Stability

Thalidomide stock solutions, stored at room temperature and 3–8°C, showed a degradation of 5–14% within a one-week period whereas phenacetin stock solutions were found to have maximal stability for at least 27 days both at room temperature as well as at 3–8°C.

Thalidomide in serum samples stored at room temperature were found to be stable (<5% degradation) for at least 2 h. A 4-h period after preparation, 66% of the initial thalidomide concentration was found. Thalidomide in serum at 3–8°C was stable (<5% degradation) for at least 5 h. After 70 h <25% thalidomide was found again in serum stored at both temperature conditions. Storing the serum samples at −20°C resulted in maximal stability of thalidomide for six days (>95%) (Fig. 3). After this period of time a larger decrease of the thalidomide concentration was observed, with a 22% loss over a period of 10 days.

Samples after protein precipitation with trichloroacetic acid were found to be stable for a period of at least 71 h after serum clean-up.

3.2. Serum concentration curve of thalidomide in a human volunteer

The maximal serum concentration was reached 8 h after drug administration. The maximal concentration was found to be 0.62 mg/l. An elimination half-life of 11.1 h was calculated from the serum concentration–time curve (Fig. 4).

4. Discussion and conclusion

We developed a new method for the determination of thalidomide in human serum, using protein precipitation as an alternative for the conventional liquid–liquid extraction methods. This method has the advantage of being fast and practical, which makes it feasible for the determination of labile compounds, such as thalidomide. The precipitation reagent, trichloroacetic acid, forms nonsoluble salts with proteins, which rapidly precipitate. After centrifugation, the aliquot can directly be injected into the chromatographic system. Apart from the fast deproteination of serum, trichloroacetic acid also prevents the hydrolytic degradation of thalidomide. Therefore, the addition of other chemicals to stabilize thalidomide can be circumvented. In contrast with previous published assays, the presented method prevents thalidomide degradation during storage and during the entire analysis. Our method does not account for the chiral separation of thalidomide
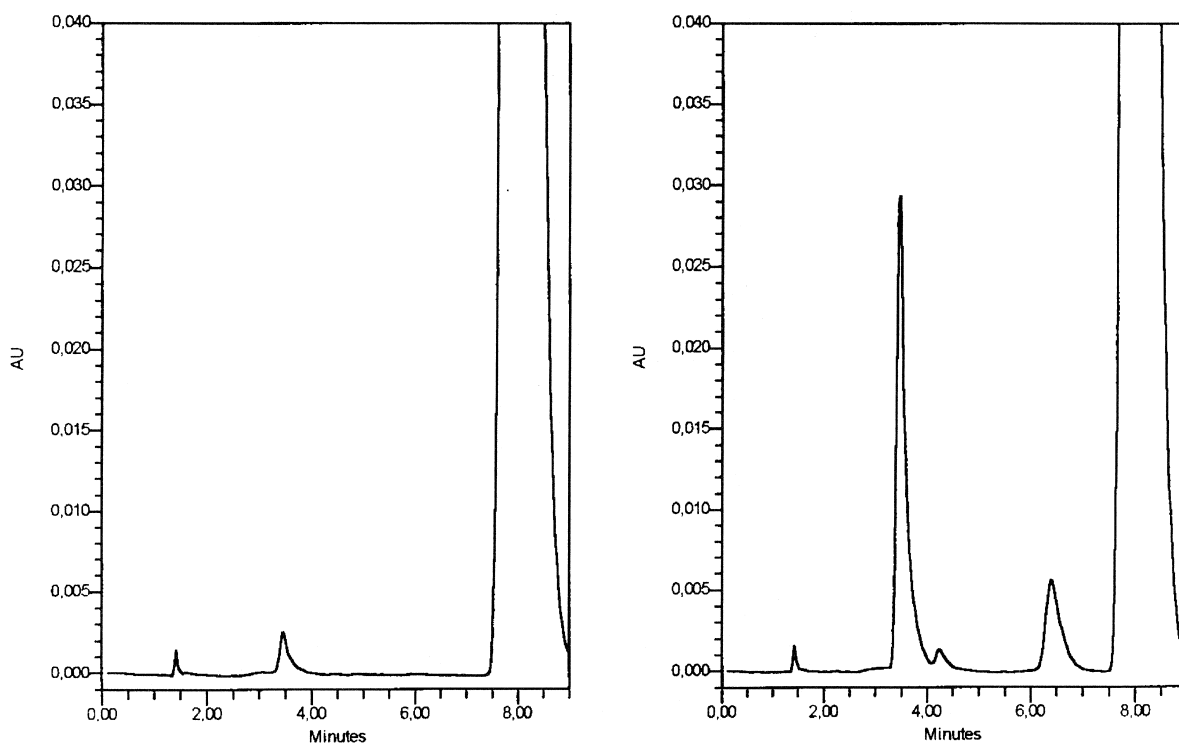
Fig. 3. Chromatograms of a freshly prepared thalidomide stock solution (A) and the same solution after storage for six days at 3–8°C (B).

enantiomers. Wnendt et al. have described a method which enables one to quantitate both enantiomers [6]. However, at the low serum concentrations used in our study (~0.2–1 mg/l) no significant differences on TNF-α inhibition and sleep inducing effects among the two enantiomers were observed [6] which makes our method suitable for our studies.

Thalidomide in serum was found to be stable for 5 h at 3–8°C and six days at −20°C, which allows transportation of human samples, immersed in an ice bath, to the laboratory and storage at −20°C until analysis without significant degradation of thalidomide. Thalidomide remained stable for at least 2 h at room temperature which provides sufficient time from defreezing up to sample work-up. After sample preparation, thalidomide, in combination with trichloroacetic acid, was found to remain stable for a practicable period of time for serial chromatographic analysis. Hydrolysis during chromatography was prevented by acidification of the eluent. Following this protocol allows samples to be quantified with high precision under practicable conditions.

In Fig. 3, a chromatogram of a freshly prepared

thalidomide stock solution is compared with a chromatogram of the same solution after six days storage at 3–8°C. The freshly prepared thalidomide solution seems to start degrading immediately after dissolving, since a peak separately from the thalidomide peak, with the same characteristic UV spectrum as thalidomide, appears at a retention time of 3.5 min. After a few days even more degradation products at retention times of 4.3 and 6.4 min can be detected in addition to the increased peak of the initial degradation compound. These compounds are more hydrophylic than the parent compound and chromatograph faster. None of these compounds interfere with the assay of the parent compound, as they chromatograph separately from the thalidomide peak and as the peak height of the detectable degradation compounds is much smaller compared with the thalidomide peak height. To ensure a correct chromatographic separation and to avoid possible miscalculation due to decreased concentrations, stock solutions of thalidomide should be freshly prepared for quantitation.
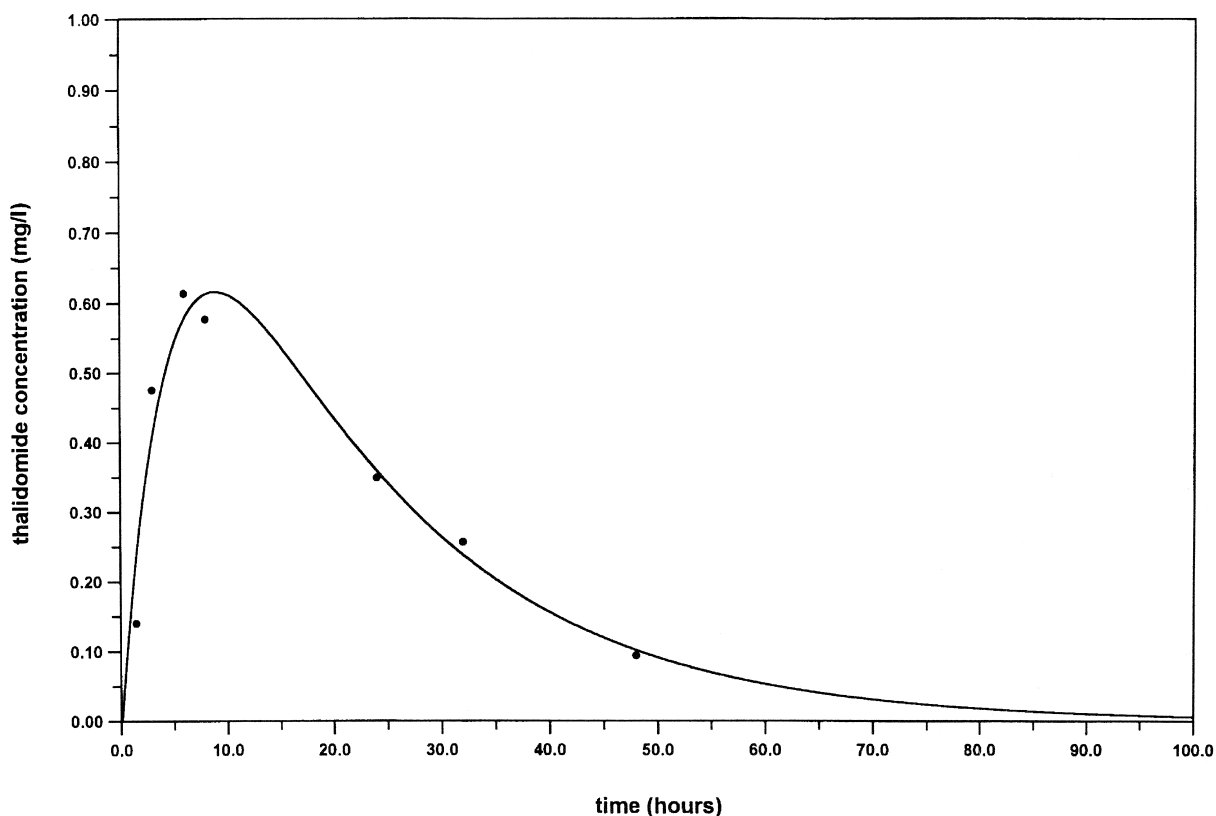
The bioanalytical assay is now applied to a

Fig. 4. Serum concentration–time curve of thalidomide in a human subject, given 400 mg orally.

pharmacokinetic study in patients receiving thalidomide and appeared to be feasible for this purpose.

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