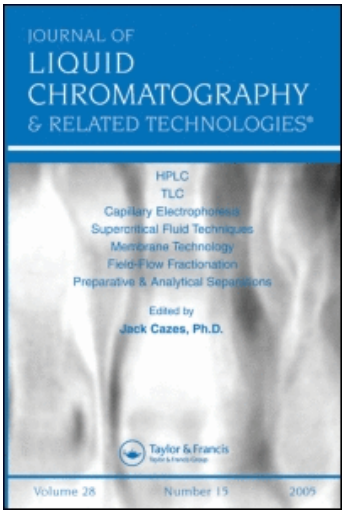

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Rapid and Sensitive Determination of Thalidomide in Human Plasma by High Performance Liquid Chromatography

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RAPID AND SENSITIVE DETERMINATION OF THALIDOMIDE IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A sensitive and rapid High-performance liquid chromatographic method using U.V.detection, has been developed for the analysis of thalidomide in plasma. This involved a single liquid-solid extraction on Extra-Sep-C8 column in the presence of an internal standard (ciprofloxacin). Analysis was performed by isocratic elution with a mobile phase consisted of 0.01 M aqueous potassium dihydrogen phosphate containing 21 % (V/V) acetonitrile and 4.5 mM Heptane sulfonic acid, adjusted to pH 2.3, with U.V detection at 295 nm. The limit of sensitivity of the assay was 0.06 mg/l. The method was applied to a pharmacokinetic study (50 to 100 mg)in patients with erythema nodusum leprosum (ENL) with a good accuracy (96-111 %) and precision (less than 5.8 %).

INTRODUCTION

Thalidomide (α- phtalimidoglutarimide) was introduced as a non-barbiturate hypnotic in 1956, and was withdrawn from the market in 1961 because of teratogenic effects (1). Its immunosuppressive properties have been known and Thalidomide

has been used in different immunologically mediated diseases including lepromatus leprosy, aphtous stomatitis, and discoid lupus erythematosus for over twenty years (2,3).

Recently, this drug was proposed in the treatment of acute and chronic graft-versus-host disease (GVHD) in bone marrow transplant patients (4).

Several analytical methods using colorimetric detection (5), UV spectrometry (6), paper and thin layer chromatography (7,8) have been reported for thalidomide but these procedures suffer from a lack of selectivity and/or sensitivity. A Gas chromatography method was described but accuracy and precision were not indicated (9). Several H.P.L.C methods have been recently proposed. However two H.P.L.C methods have been developped (10,11) but they are characterized by rather poor sensitivity with achieving detection limit of only 1.0 mg/l.

Another H.P.L.C assay was proposed (12) with a reported sensitivity near 0.1 mg/l, but no data was available concerning accuracy and precision. Recently, a sensitive, specific, and accurate H.P.L.C procedure was described (13) but the sample work-up procedure using a liquid-liquid extraction was time-consuming thereby hampering extended pharmacokinetic study with large numbers of samples. In this paper, we describe a rapid, accurate and sensitive method for the quantitative determination of thalidomide in plasma by H.P.L.C using reversed-phase chromatography, solid phase extraction and U.V. detection.

MATERIALS AND METHOD

Chemicals

Thalidomide was kindly supplied by Dr Frankus from Chemie Grünenthal (Aachen, Germany) and ciprofloxacin (internal standard) by Bayer-Pharma Laboratories (Sens, France).
H.P.L.C grade acetonitrile, methanol and dimethylformamide, RPE-ACS grade potassium phosphate monobasic and sodium dihydrogen phosphate were obtained from Carlo-Erba (Milan,

Italy) and used for the preparation of an 0.066M aqueous phosphate buffer. 1-Heptane sulfonic acid (H.S.A) was from Sigma (Saint Louis, U.S.A).

Analytical grade orthophosphoric acid, hydrochloric acid and di-sodium hydrogen phosphate 2-hydrate were from Merck (Darmstadt , Germany). Extraction columns (Extra-Sep C_8, 200 mg , 3 ml, Lida Manufacturing Corp.,Kenosha, U.S.A) were from Touzart-Matignon (Paris, France). Ultrapure water was obtained before use, through a Milli Q- plus water purification system (Millipore Corp.,U.S.A).

Chromatography

H.P.L.C system consisted of a model 510 Waters pump, a model 231 Gilson autosampler unit with a 100 µl loop, models 484 Waters U.V. absorbance detector and 746 integrator-recorder.The column (150 x 4.6 mm i.d) was packed with Nucleosil C_8, 5 µm particule size (Touzart-Matignon, France). The mobile phase consisted of 0.01M aqueous potassium dihydrogen phosphate solution containing 21 % (V/V) acetonitrile and 4.5 mM H.S.A, adjusted to pH 2.3 with concentrated orthophosphoric acid.

Prior to use, the mobile phase was filtered through a H.V.L.P 04700 Durapore membrane (Millipore Corp.,U.S.A). This, was carried through the column at 0.8 ml/min and the detection wavelength was set at 295 nm. All separations were carried out at room temperature.

Standard solutions

The stock solution (1000 mg/l) of thalidomide was prepared weekly in dimethylformamide, and stored at + 4°C. The stock solution (100 mg/l) of ciprofloxacin (I.S.) was weekly prepared in methanol and stored in similar conditions.

The working solutions were prepared by dissolving the stock solution in 0.01N HCl, at final concentrations of 10.0- 5.0 - 2.5 - 1.25 - 0.625 mg/l for thalidomide, and 2.0 mg/l for I.S.

Calibration standards : (0 - 0.0625 - 0.125 - 0.25 - 0.5 - 1.0 mg/l) were made daily from pooled human plasma. These were prepared according to the sample preparation procedure.

Sample preparation

The solid-phase extraction column (Extra-Sep C_8) was conditioned by elution with 3 x 1 ml of 0.066M phosphate buffer. Plasma sample (1.0 ml) supplemented with 60 µl of I.S. methanolic solution (2.0 mg/l) and mixed with an equal volume of 0.066M pH 7.4 phosphate buffer, was loaded onto the conditioned column. This fraction was washed with 3 x 1 ml of 0.1M HCl. Both thalidomide and I.S. were recovered with 5 x 0.2 ml of dimethylformamide. The eluate was evaporated to dryness at ambient temperature under a gentle stream of nitrogen gas. The dry residue was reconstituted in 130 µl of mobile phase and an aliquot of 100 µl was injected into the chromatographic system.

RESULT AND DISCUSSION

Analytical conditions

Solid - phase extraction (SPE) provides fast and efficient sample preparation, it reduces sample handling and eliminates the risk of emulsification (13,14). The use of ciprofloxacin as I.S. improves the reproducibility of the method (Table 1).

Two U.V. detection peaks are available for the analytical determination of thalidomide in biological fluids. Several authors (11,12,15) have selected a wavelength below 254 nm for U.V. detection, which gives a good sensitivity but in contrast a poor specificity.

We have selected a wavelength at 295 nm as CZEJKA M.J et al (10), which optimizes the specificity of the assay (Figure 1).

Linearity

Standard curves were constructed by plotting the peak-area ratio of thalidomide to the internal standard versus the concentration of thalidomide.

The calibration curve was linear over the selected following range (0.0625 - 1.0 mg/l) in plasma, and characterized by the equation : $y = 1.294\ x - 0.010$, ($r = 0.9994$, $n = 6$).

TABLE 1

Within-day Variability an Accuracy in Measured Thalidomide Concentrations in Plasma.

Conc. spiked (mg / l)	Conc. found (mg / l) mean ± S.D.	Accuracy (%)	Coefficient of variation (%)
0.125 (n = 8)	0.125 ± 0.006	100.0	4.8
0.50 (n = 7)	0.494 ± 0.039	98.8	7.8
1.0 (n = 7)	0.953 ± 0.070	95.3	7.3

Recovery

The absolute recovery of the SPE was determined by comparing the peak areas of thalidomide obtained from freshly prepared spiked plasma sample extracts to those obtained from aqueous standard solutions with the same concentration. The recovery was 79.5 ± 4.2 % at 0.5 mg/l and 80.3 ± 4.6 % at 1.0 mg/l, n =3

Chromatograms

Figure 1 shows chromatograms obtained with extracts from plasma spiked with 0.5 mg/l thalidomide (Figure 1a), blank plasma (Figure 1b), and plasma sample obtained 4 hours after the administration of a 100 mg daily oral dose of thalidomide, to an erythema nodusum leprosum patient (Figure 1c).

Concentrations were calculated by comparing the ratio of peak areas of samples (major peak/ I.S), with these of the calibration curve made daily. Thalidomide and I.S. were eluted in 10.6 and 16.2 min., respectively.

Limit of quantitation

The limit of quantitation for thalidomide was 0.0625 mg/l (Figure 2), when 1.0 ml of plasma was used.

The limit was taken as a chromatographic peak four times higher than baseline noise; it was always the lowest point of the calibration curve, giving good accuracy (111 %) and precision (C.V = 5.8 % , n = 6). This limit of quantitation was sufficient for pharmacokinetic studies.

Accuracy and precision

The within-day variability of the method was characterized by coefficients of variation lower than 7.8 % (Table 1).

The inter-day variability in plasma was assessed over thirty days from four series of human plasma samples containing 0.0625, 0.125, 0.50 and 1.0 mg/l of thalidomide (Table 2). The C.V for inter-day analysis was less than 6 % and the accuracy inside a 99.2 - 111 % range.

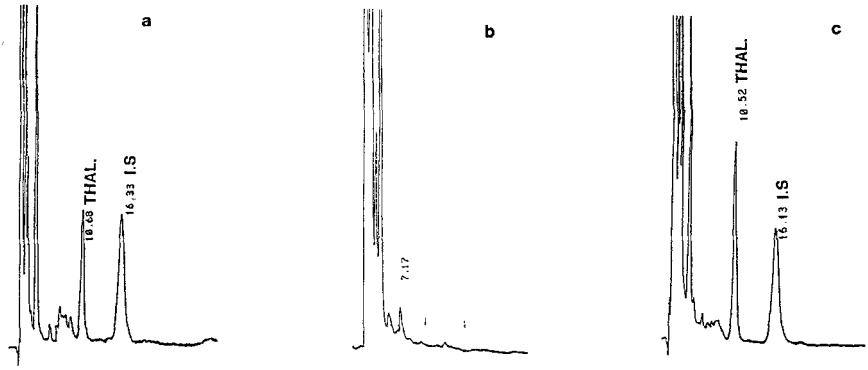

FIGURE 1 : Chromatograms obtained from extract of

(a) plasma spiked with thalidomide (THAL.)(0.5 mg/l) and internal standard (I.S)

(b) human blank plasma

(c) plasma sample obtained at the 4 th hour from a patient included in a pharmacokinetic study : THAL. : 0.8 mg/l

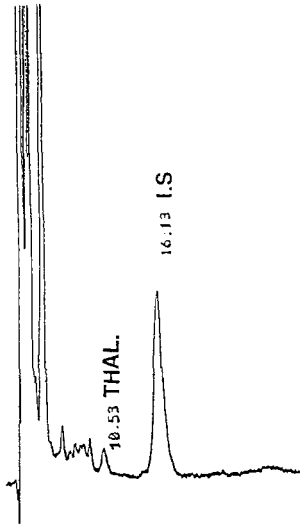

FIGURE 2 : Chromatogram obtained from extract of a blank plasma spiked at the limit of quantitation : 0.06 mg/l of THAL.

TABLE 2

Between-day Variability and Accuracy in Measured Thalidomide Concentrations in Plasma.

Conc. spiked (mg / l)	Conc.found (mg / l) mean ± S.D	Accuracy (%)	Coefficient of variation (%)
0 (n= 6)	N.D.	-	-
0.0625 (n = 6)	0.0697 ± 0.004	111.0	5.8
0.125 (n = 6)	0.120 ± 0.007	96.0	5.8
0.5 (n = 6)	0.528 ± 0.025	106.0	3.8
1.0 (n = 6)	0.993 ± 0.056	99.3	5.0

N.D. = Not Detected

TABLE 3

Retention Time for Different Drugs Administered in Patients Receiving Thalidomide Treatment.

Drug	Retention time (min)
ciclosporine	N.D
ciclophosphamide	N.D
prednisolone	N.D
hydroxyzine	N.D
nifédipine	N.D
diltiazem	N.D
amphotéricine B	N.D
clonazépam	N.D
clobazam	N.D
diazépam	N.D
desmethyl-diazépam	N.D
acyclovir	2.0
flucytosine	2.5
métronidazole	3.0
azothioprine	4.4
ceftazidime	4.5
céfotaxime	8.0
thalidomide	10.6
ciprofloxacine	16.3

N.D = Not Detected

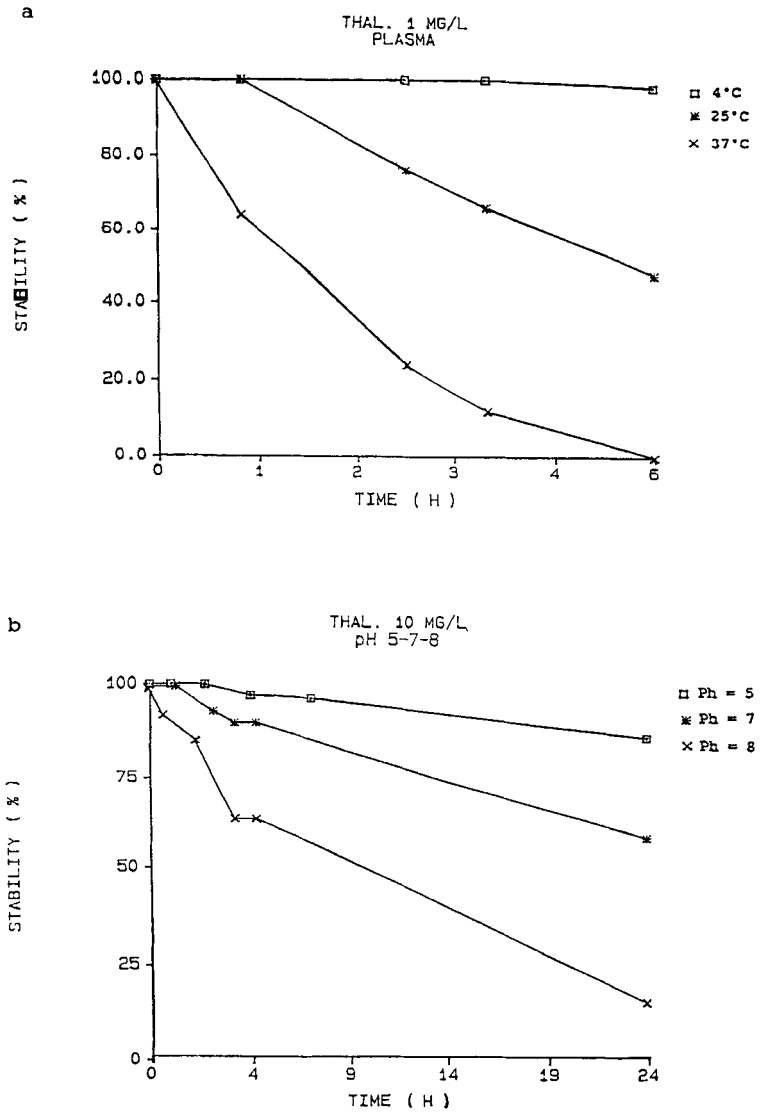

FIGURE 3 : Curves of thalidomide stability

(a) plasma extracts at different temperature conditions.

(b) in different pH conditions of aqueous buffer.

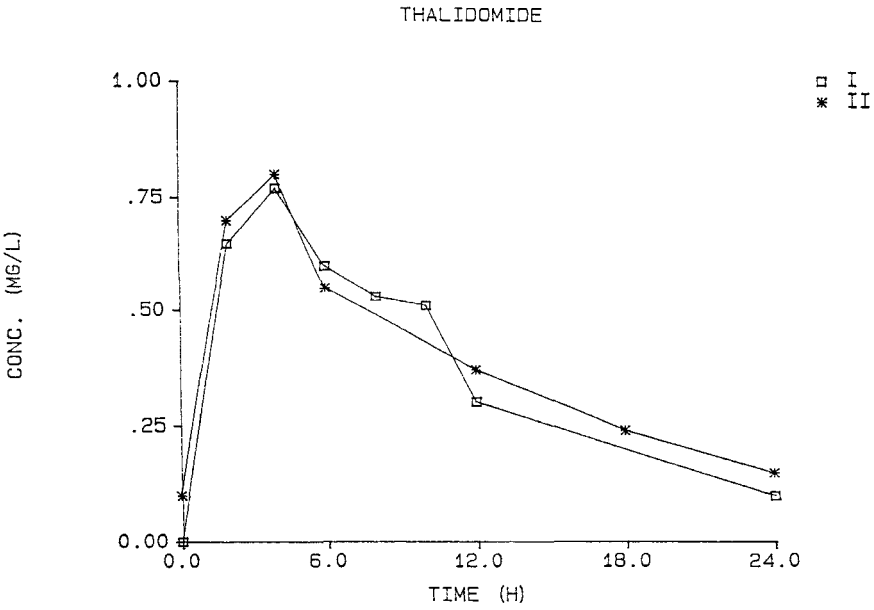

FIGURE 4 : Plasma levels of thalidomide with time following single dose oral administration (100 mg) in patient I, and at steady-state (100 mg/24 h) in patient II.

Specificity

Table 3 , lists the retention times of different drugs potentially administered to patients receiving thalidomide. Most were not detected, and no interferences appeared when peaks occurred on chromatograms. Their retention times were sufficiently different from those of thalidomide and I.S. to preclude any chromatographic interference.

Stability

Several authors reported an hydrolysis of thalidomide in aqueous medium at pH > 7 (10, 12, 14). We have investigated the influence of both temperature and pH on its stability.

Thalidomide and ciprofloxacin (I.S) were stable in stock solutions for at least a week in the refrigerator. A good stability was observed for at least 6 h at + 4°C and 1 h at 25°C in plasma samples (1.0 mg/l).(Figure 3 a).

No significant degradation was observed at 10 mg/l in aqueous buffer at pH 5 and kept at 25 °C. Instability appeared at pH 7 and increased in alcaline conditions (Figure 3 b).

Thalidomide stability was verified in both 0.066M phosphate buffer (pH 7.4) and H.P.L.C injection solvent for at least 25 min. and 12 h, respectively.

Pharmacokinetic application

The applicability of this assay was examined in a preliminary pharmacokinetic study in erythema nodosum leprosum patients.

The plasma concentration-time curves of thalidomide are shown in figure 4. From the results of this preliminary study, it appears that this method would be suitable in plasma drug monitoring of both E.N.L patients and bone marrow recipients suffering of graft-versus-host-disease (GVHD).

CONCLUSION

The H.P.L.C assay described here showed good reproductibility, accuracy and selectivity. It is sufficently sensitive and suitable to be used in pharmacokinetic studies, and therapeutic drug monitoring.

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