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Determination of thalidomide in plasma and blood by high-performance liquid chromatography: avoiding hydrolytic degradation

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ABSTRACT

Thalidomide was determined in plasma and blood by reversed-phase high-performance liquid chromatography with ultraviolet detection. The coefficient of variation of the assay was 1–2% over the 0.25–4.0 μg/ml concentration range. Hydrolysis of thalidomide during storage and work-up of the samples was avoided by the addition of an equal volume of citrate buffer, pH 1.5. The assay could be applied to the determination of blood concentrations of thalidomide in rats for at least 28 h after a single oral dose, with multiple blood sampling from the same animal.

INTRODUCTION

Thalidomide was introduced as a sleep-inducing agent in 1956. It was withdrawn in 1961 for causing foetal abnormalities. Later thalidomide has been tested in various immunological diseases and has been re-introduced as an immunomodulating drug, *e.g.* in treating and preventing graft-*versus*-host disease, and as an anti-inflammatory agent, notably in the lepra reaction [1].

Chromatographic assays for thalidomide in biological samples have been described [2–5]. A potentially useful gas chromatographic (GC) method was reported already in 1969, but no data on accuracy or precision were given [2]. Czejka and Koch [3] determined thalidomide in human serum by high-performance liquid chromatography (HPLC), but the dynamic range of their assay was 1–20 μg/ml, which is well above the plasma concentrations of 0.2–1.4 μg/ml attained during the first 24 h after a single therapeutic dose [4]. The HPLC assay used in the latter study had a sufficiently low linear range of 0.1–10 μg/ml. For a third HPLC method [5], linear range, accuracy and precision were not reported.

Very importantly, only in the early GC assay [2] account is taken of the rapid spontaneous hydrolysis of thalidomide in any aqueous medium at physiological pH [6–8]. The purpose of this work was therefore to create a rapid and sensitive HPLC assay for thalidomide in plasma and blood and to establish the prevention of hydrolytic degradation of thalidomide in samples and stock solutions.

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EXPERIMENTAL

Reagents and chemicals

Thalidomide was kindly supplied by Grünenthal (Stolberg/Rheinland, Germany), and phenacetin was of European Pharmacopoeia quality. All stock solutions were prepared in methanol and kept in the freezer. Diethyl ether was purchased from BDH (Poole, UK) and acetonitrile from E. Merck (Darmstadt, Germany). Methanol was of US Pharmacopoeia quality. Water was freshly distilled and collected in a stainless-steel vessel.

Instrumentation and chromatographic conditions

The HPLC system consisted of an LDC/Milton Roy (Riviera Beach, FL, USA) Constametric III pump, a Rheodyne 7125 loop injector with a 20-μl loop and a Spectromonitor III variable-wavelength UV detector. A LiChrocart (Merck) RP-18, 7-μm column (250 mm × 4 mm I.D.) was used, and the mobile phase was a mixture of acetonitrile–water (35:65). The flow-rate was 1.2 ml/min and the detection wavelength was 220 nm.

Samples

Blood samples were drawn from Wistar Furth rats who received 50 mg/kg thalidomide in a suspension, as a single oral dose. The suspension was made freshly by adding a solution of Polysorbate 80–water (1:99) to thalidomide and then administered via a gastric tube. Blood samples were taken from a catheter inserted into a femoral artery and tunnelated to the neck. Blood, approximately 0.5 ml, was withdrawn into a heparinized syringe and the sample to be analysed was then collected into Blodcaps glass capillaries (100 or 200 μl) by spontaneous outflow from the catheter. The initially drawn blood was re-injected followed by 1 ml of heparin-saline (50 U/ml). Each blood sample was directly transferred to a glass-stoppered tube containing an equal volume of 0.025 *M* Sörensen's citrate buffer (pH 1.5). The blood–buffer mixtures were stored at −25°C until analysis, for a maximum of 4 days.

The actual volume measured with the glass capillaries was checked by weighing ten capillaries of each nominal volume empty as well as filled with either water or human blood.

Sample work-up

To each sample (0.2–2.0 ml of diluted blood or plasma) were added 50 μl of internal standard solution (100 μg/ml phenacetin) and 5.0 ml of diethyl ether, and the mixture was vortex-mixed for 30 s. The solvent layers were separated by centrifugation at 1200 *g* for 15 min, and the organic layer was transferred to another tube. The solvent was evaporated under a stream of dry air, the residue was taken up in ten drops of mobile phase, and 20 μl of this solution were injected into the chromatograph.

Due to the risk of interference in the chromatograms, the use of possible sources of contaminants, *e.g.* plastic ware, detergents and community water, was avoided. Glass-stoppered tubes washed with distilled water and 99% ethanol were used throughout.

Standard curves and precision

Standard curves were prepared from 1 ml of plasma–buffer or blood–buffer solution (1:1), spiked with 5 μg of internal standard and 0.125–2.0 μg of thalidomide. Precision in plasma–buffer and blood–buffer samples was checked by assaying eight samples (1 ml) spiked with 0.125 or 2.00 μg of thalidomide (*i.e.* 0.250 or 4.00 μg/ml of plasma or blood).

Extraction yields

The extraction yields of thalidomide from plasma–buffer, blood–buffer and normal plasma were determined using four samples of each, spiked with 1 μg/ml thalidomide. After the work-up, 5 μg of phenacetin were added as external standard for quantitation. The extraction yield of phenacetin was determined analogously.

Stability of thalidomide during sample work-up

The stability of thalidomide in normal plasma during the sample work-up was checked using 0.5-ml plasma samples spiked with 5 μg of phenacetin and 0.5 μg of thalidomide. Three samples were worked up and injected as fast as possible,

three were left on the bench for 2 h before extraction, three for 2 h before centrifugation, three for 3 h after evaporation of the solvent and three for 3 h after taking up in mobile phase.

Stability of thalidomide in stock solutions

Degradation of 100 μg/ml thalidomide in methanol at room temperature (23°C), in the refrigerator (5°C) and in the freezer (−25°C) was determined at 5, 9, 19, 99, 139 and 176 days by direct injection into the chromatograph, with comparison with freshly made solutions.

Stability of thalidomide in normal and acidified plasma and blood

Thalidomide was added to freshly drawn, heparinized human blood or plasma to a concentration of 1.0 μg/ml. The mixtures were kept at 23°C in the dark. Duplicate samples were taken at 0, 1, 2, 4, 6.5, 22, 26 and 31 h. An equal volume of citrate buffer solution was added, and the samples were stored at −25°C until analysed (within 4 days). The pH of the spiked blood and plasma was checked repeatedly during the incubation, using an ABL 3300 automatic blood gas laboratory (Radiometer, Copenhagen, Denmark). The experiment was repeated with mixtures kept at 5°C and seven duplicate samples taken over 96 h.

In addition, thalidomide was added to plasma–buffer and blood–buffer solutions (1:1) to a concentration of 0.5 μg/ml. Each mixture was portioned into sixteen samples that were kept at 23°C. Duplicate samples were put in a −70°C freezer at 0, 1, 5, 8, 14, 20, 26 and 32 days and stored until analysed (7 days after last sample).

Twelve 0.5-ml samples of thalidomide, 1.0 μg/ml, in fresh human blood and twelve in plasma were prepared. Six of each were assayed immediately and the remaining ones after 34 days at −25°C. They were assayed against the same stock solution of thalidomide in methanol, that had also been stored at −25°C.

Calculations

The half-lives ($t_{1/2}$) of degradation of thalidomide under various conditions were calculated by fitting monoexponential curves to the "percentage remaining" *versus* time data points, using the RSTRIP software (MicroMath, Salt Lake City, UT, USA). The rate of degradation at −25°C in blood, plasma or methanol was estimated using the Arrhenius plot; the rate constants at 23 and 5°C were plotted (on a logarithmic scale) against 1/(absolute temperature, °K) and the line was extrapolated to 1/248 °K.

RESULTS

Chromatography

Representative chromatograms are given in Fig. 1. The capacity factors (k') of thalidomide and phenacetin were 1.9 and 2.8, respectively.

Samples

The pH of blood was 5.1 after mixing (1:1) with citrate buffer solution, and that of plasma was 4.2. The volumes of the 100- and 200-μl Blodcaps, as determined by weighing of water, were 98.6 ± 0.8 and 196.7 ± 0.9 μl, respectively (mean ± S.D.). The corresponding values for

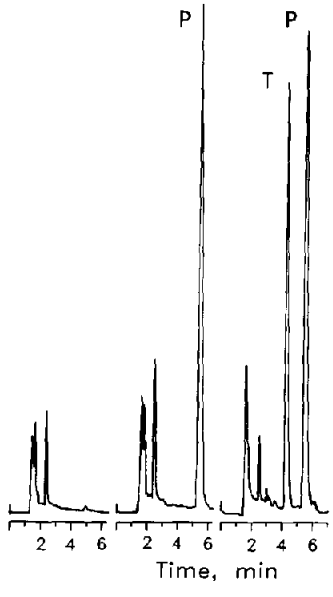

Fig. 1. Chromatograms of rat blood samples. From left to right: blood (0.20 ml) taken before administration of thalidomide; a duplicate sample with internal standard (phenacetin 5.0 μg) added; and a sample (0.60 ml) taken 22 h after the administration of 50 mg/kg thalidomide orally. The thalidomide concentration is 1.96 μg/ml. Peaks: P = phenacetin; T = thalidomide.

blood, after correction for the density of whole blood, were 98.2 ± 0.8 and 196.7 ± 1.5 μl.

Standard curves and precision

The standard curves were linear (generally r = 0.999) up to thalidomide concentrations of 4 μg/ml. Standard curves from blood–buffer and plasma–buffer were superimposable. The coefficient of variation (C.V.) in plasma–buffer solution was 1.1% at 125 ng (found 127 ± 1.4 ng) and 2.3% at 2.0 μg (found 2.00 ± 0.046 μg). Corresponding values in blood–buffer solution were 1.1% at 125 ng (found 123 ± 1.4 ng) and 0.8% at 2.0 μg (found 2.02 ± 0.016 μg) (n = 8 in all determinations).

Extraction yields

The extraction yield of thalidomide from acidified plasma was 93% and from acidified blood 87%. The corresponding values for phenacetin were 87 and 86%. The extraction yield of thalidomide from normal plasma was 96%.

Stability of thalidomide during sample work-up

There was a 25% loss of thalidomide in normal plasma left for 2 h before extraction. In the remaining steps no degradation was detected. Extracted and reconstituted samples could be stored for 4 days at −25°C without apparent degradation.

Stability of thalidomide in stock solutions

Degradation of thalidomide in methanol (at 23 and 5°C) was indicated by peaks at k' = 1.05, 1.52 and 1.62. There were no such peaks in the chromatograms when solutions kept at −25°C were injected, not even after 176 days. Comparison with freshly made solutions did not indicate any significant decrease in concentration. The half-lives of thalidomide in methanol at 23 and 5°C are given in Table I.

Stability of thalidomide in normal and acidified plasma and blood

The measured and estimated half-lives of thalidomide in the various media are given in Table I. The pH of the freshly drawn venous blood was 7.1. It rose to 7.7 in 5 h and then declined slowly to 7.3 at 31 h. The pH of the plasma rose quickly to 7.6 and remained constant during the first day of the experiment. At 31 h it had declined to 7.2.

Storing spiked blood samples, without citrate buffer, for 34 days at −25°C resulted in a 13% degradation, which corresponds to a $t_{1/2}$ of approximately 170 days. The values for plasma were 15% degradation and $t_{1/2}$ approximately 145 days.

TABLE I

RATES OF DEGRADATION OF THALIDOMIDE IN METHANOL, HUMAN BLOOD AND PLASMA

Medium	Temperature (°C)	Half-life
Methanol	23	44 days
	5	217 days
	−25	12 years[a]
Blood	23	11 h, 13 h[b]
	5	82 h
	−25	125 days[a]
Plasma	23	7.0 h, 6.7 h[b]
	5	68 h
	−25	222 days[a]
Blood–buffer	23	13 days
Plasma–buffer	23	44 days

[a] Estimated from the Arrhenius plot.
[b] Two experiments.

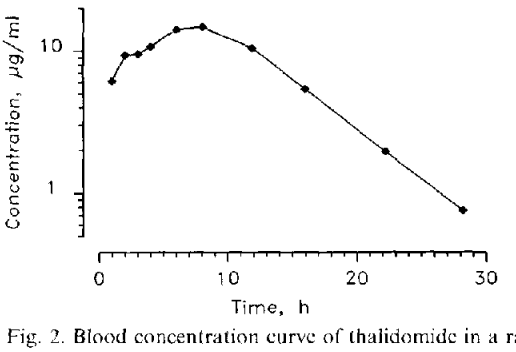

Fig. 2. Blood concentration curve of thalidomide in a rat given 50 mg/kg orally, as a suspension in Polysorbate 80 water. Duplicate blood samples were taken at each time point, with volumes ranging from 100 μl generally to 600 μl at the penultimate sampling and 1.0 ml at the end of the experiment.

Blood concentration curve of thalidomide in one rat

A representative blood concentration curve is depicted in Fig. 2.

DISCUSSION

Our data confirm the previously reported [6–8] hydrolytic degradation of thalidomide in aqueous media at physiological pH. The recent claim [4] that binding of thalidomide to plasma proteins would afford sufficient protection from hydrolysis was not supported. Indeed, pharmacokinetic data, obtained using tritiated thalidomide in rats and rabbits [7] or rhesus monkeys [8], suggest that non-enzymatic hydrolysis of thalidomide contributes markedly to its *in vivo* clearance.

The rate of hydrolysis of thalidomide is very sensitive to changes in pH within the physiological range [6]. Consequently, minor variations in pH can sufficiently explain the differences in $t_{1/2}$ of thalidomide in blood and plasma and also the somewhat poor reproducibility of the data. Establishing a "true" rate of hydrolysis of thalidomide in blood or plasma would require a careful control of pH. In untreated samples, the pH is not well controlled. Arterial blood normally has a pH of 7.4–7.5, but venous blood is more acidic, due to a higher concentration of carbon dioxide. When this evaporates, the pH will rise.

Below pH 6, thalidomide is rather stable [2,6], and acidifying the blood or plasma with citrate buffer accordingly decreased the rate of degradation by more than one order of magnitude. This should provide sufficient protection against degradation during work-up and also permit the storage of samples for a month at −25°C. Citrate, in contrast to heparin, is also a good anticoagulant for rat blood.

The estimates of the rates of degradation at −25°C should also be taken as approximate. The Arrhenius plot is based on the assumption that, apart from the change in temperature, all conditions remain constant. Freezing (*i.e.* solidifying) the sample of course violates this in the case of plasma and blood. Still, the degradation of thalidomide observed with storage of the untreated blood or plasma samples for 34 days at −25°C is in reasonable agreement with the half-lives estimated by means of the Arrhenius plot. The $t_{1/2}$ of thalidomide in methanol at −25°C corresponds to a 3% degradation in 6 months, which sets a convenient time limit for storage.

The first HPLC assay for thalidomide [3] also aimed to determine the hydrolysis products. This necessitated the use of an ion pair-forming agent in the mobile phase and a rather time-consuming separation on the column. Even when the system was simplified for the assay of unchanged thalidomide only, the chromatography of the plasma extract took more than 15 min. Chen *et al.* [4] used a methanol–buffer gradient taking 12 min: the large peaks (or baseline drifts) that appear in their chromatograms by that time presumably result in a considerably longer interval between injections. Heney *et al.* [5] used a simple isocratic system similar to ours, but chromatography of a sample took 19 min. In contrast, our method permits a sample injection every 6 min.

The need for a comparatively polar organic solvent to extract thalidomide from acidified plasma of blood, in combination with low-wavelength UV detection, inevitably makes an assay like this sensitive to interfering compounds. This has been noted previously [5]. The sampling of arterial blood by spontaneous outflow from the catheter in the rat experiments was designed to avoid contacts with syringes or other potentially contaminating surfaces, albeit originally for another assay [9]. Correct washing and rinsing of glassware is imperative. We also chose to avoid screw-cap tubes, using the same glass-stoppered tube for sample collection and extraction.

We showed the applicability of the assay to studies in rats. The assay has sufficient accuracy and precision in the plasma concentration range attained by thalidomide in human pharmacokinetic studies.

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