A New Method for Determination of Both Thalidomide Enantiomers Using HPLC Systems

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Although thalidomide was withdrawn due to teratogenicity and neuropathy, there is now growing clinical interest in this compound because of its immunomodulatory and anti-angiogenic properties. In 1998, thalidomide was approved by the U.S. Food and Drug Administration for the treatment of erythema nodosum leprosum (ENL), an inflammatory complication of Hansen’s disease, through a restricted-use program. Thalidomide was approved for the treatment of relapsed or refractory multiple myeloma (MM) as an orphan drug in Japan. Direct deproteinization method was shown to be useful for quantitation of enantioselective thalidomide blood level. Stabilized blood was deproteinized with methanol and 2 M trichloroacetic acid. The supernatant was injected onto reverse-phase column (CHIRALPAK AD-RH). The mobile phase consisted of 10% acetonitrile, 70% methanol and 20% 0.025 M citrate buffer (pH 3.0), and the flow rate was 0.5 ml/min. Wavelength of detection was 220 nm. (−)-(S)-thalidomide and (+)-(R)-thalidomide were separated at 13.5 min and 17.6 min, respectively. The accuracy of this method was almost the same as that of the measurement technique with extraction and concentration. In clinical practice, MM patients usually take many kinds of drugs at the same time. Actually, this patient takes a lot of drugs with thalidomide. However, we found no interference of these drugs and thalidomide on the chromatogram. This simple and reliable HPLC determination method for both enantiomers of thalidomide is thought to be very useful for thalidomide studies.

Key words thalidomide; enantiomer; multiple myeloma; simultaneous separation

Thalidomide (Fig. 1) was developed in the 1950s as a sedative having low toxicity. However, in 1961 McBride and Lenz reported a close correlation between oral administration of thalidomide in pregnant women and a particular deformity (phocomelia) of their babies.1,2 Although thalidomide was withdrawn due to teratogenicity and neuropathy, there is now growing clinical interest in this compound because of its immunomodulatory and anti-angiogenic properties. Current evidence indicates that thalidomide reduces the activity of the inflammatory cytokine tumor necrosis factor (TNF)-α by accelerating the degradation of its messenger RNA.3,4 Moreover, D’Amato et al. suggested a utility of thalidomide as a therapeutic agent for diseases that involve angiogenesis, particularly tumorous diseases.5 In 1998, thalidomide was approved by the U.S. Food and Drug Administration for the treatment of erythema nodosum leprosum (ENL), an inflammatory complication of Hansen’s disease, through a restricted-use program.5 Recently, some investigators have reported that thalidomide is efficacious against human immunodeficiency virus (HIV)-associated wasting syndrome, HIV-related diarrhea, oral aphthous ulcers and Kaposi’s sarcoma.7 Its immunomodulatory, anti-inflammatory, and antiangiogenic properties are currently under study in a number of clinical conditions.8–10 Furthermore, in 1994, Vacca et al. reported that the bone marrow of multiple myeloma (MM) patients was rich in blood vessels and that there is a causal relationship between the activity of MM and marrow angiogenesis.11 In 2005, thalidomide was approved for the treatment of refractory MM as an orphan drug in Japan. The thalidomide product is a racemic mixture of (−)-(S)- and (+)-(R)-enantiomeric forms of a synthetic glutamic acid derivative. (−)-(R)-Thalidomide, but not (−)-(S)-thalidomide, exhibited significant positive influences on all sedative effects (sleep, tiredness, and reaction times). On the other hand, (−)-(S)-thalidomide had a significant effect in the opposite direction (Fig. 1). The results of several published pharmacokinetic studies are questionable due to poor methodology and the use of non-stereospecific assays.12–17 The enantiomers of thalidomide undergo spontaneous hydrolysis and fast chiral interconversion at physiological pH.12,18

In the present study, we tried to develop a new method for determination of blood levels of both thalidomide enantiomers.

MATERIALS AND METHODS

Study Japanese patient with MM was included at Sapporo Japan Post Hospital. Written informed consent was obtained from the patient when she was enrolled in this study. The study was approved by the Ethics Committee of Sapporo Japan Post Hospital.

Fig. 1. Chemical Structures of (−)-(S)-Thalidomide and (+)-(R)-Thalidomide

The asterisk (+) indicates the position of the chiral carbon.

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Chemicals A racemic mixture of thalidomide (rac-thalidomide), (–)-(S)-thalidomide and (+)-(R)-thalidomide was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile, HPLC-grade methanol, trisodium citrate, citric acid monohydrate and trichloroacetic acid were purchased from Wako Pure Chemical (Osaka, Japan). Reverse Osmosis (RO) water was obtained from an in-house Pure TRO system (Life, Tokyo, Japan).

Instrumentation and Chromatography The HPLC system was composed of a valve RHEODYNE 7125, Hitachi L-7110 pump, Hitachi L-7450 UV detector, Hitachi D-2500 chromato-integrator and Hitachi L-7300 column oven. The column was a CHIRALPAK AD-RH column (150 mm × 4.6 mm i.d., DAICECL CHEMICAL INDUSTRIES, Tokyo, Japan) with a guard column of the same type (4 mm i.d. × 10 mm). A mobile phase consisting of 10% acetonitrile, 20% methanol and 70% 0.025 M citrate buffer (pH 3.0) was used. Column temperature and flow rate were 40 °C and 0.5 ml/min, respectively. The wavelength of the detector was 220 nm.

Sample Preparation Whole blood samples were collected in heparinized collecting blood tubes. One hundred microliters of a sample was mixed with 100 microliters of methanol and 30 microliters of 2 M trichloroacetic acid were added to the thawed sample. After vortexing, the sample was centrifuged at 16400 × g for 20 min at 4 °C. Eighty microliters of supernatant was injected onto the HPLC column for analysis.

Quantitation A stock solution of thalidomide (100 μg/ml of rac-thalidomide) was prepared in methanol and then diluted to prepare a standard solution. The standard solution was diluted with a blood/citrate mixture to prepare calibration standards between 0.05 and 5.0 μg/ml. Accuracy (mean±S.D.) was determined by analysis of rac-thalidomide (0.05, 0.1, 0.2, 1.0, 5.0 μg/ml) in five replicates. The limit of quantification (LOQ) was defined as the lowest drug concentration that could be determined with acceptable precision and accuracy. The limit of detection was defined as the amount that could be detected with a signal/noise ratio of 5.

RESULTS AND DISCUSSION

Thalidomide has received much attention recently due to its clinical interest.1,2,6,7 Thalidomide is a racemate with known pharmacologic and pharmacokinetic enantioselectivity.12–17 Thus, it is important to develop an easy and effective method for determination of blood levels of both thalidomide enantiomers. We developed an HPLC method for simultaneous separation and determination of (–)-(S)-thalidomide and (+)-(R)-thalidomide in human whole blood. Although serum samples with equal volumes of citrate buffer (pH 2, 0.2 M) are stable in storage at −80 °C,19 the acidified whole blood sample with citrate buffer (pH 1.5, 25 mM) was stable in storage at −30 °C within 75 d.20 Thus, we chose the method by Eriksson and Björkman. Instead of extraction with organic solvents, we used a direct deproteinization method. We can directly inject supernatant of whole blood, serum, and plasma-acidification solution mixture to an HPLC column. It has been reported that thalidomide enantiomers separation could be achieved by using several kinds of normal phase chiral columns.21,22 We therefore selected reverse-phase chiral column CHIRALPAK AD-RH.

Chromatograms of 1.0 μg/ml (+)-(R)-thalidomide in methanol, 1.0 μg/ml (+)-(R)-thalidomide and 1.0 μg/ml (–)-(S)-thalidomide in methanol are shown in Fig. 2. Retention times for (–)-(S)-thalidomide and (+)-(R)-thalidomide were 13.5 and 17.6 min, respectively, with the preferred mobile phase consisting of 10% acetonitrile, 70% methanol and 20% 25 mM citrate buffer (pH 3.0) and flow rate of 0.5 ml/min. Fine adjustment of the mobile phase composition was not needed in our system. The baseline was extremely stable. The calibration standard curves for thalidomide enantiomers had a coefficient of determination (r2)>0.999. Linearity ranged from 0.05 to 5.0 μg/ml thalidomide in samples, i.e., 0.025—2.50 μg/ml of each enantiomer. The precision for human whole blood samples is summarized in Table 1. LOQ of both thalidomide enantiomers was 0.025 μg/ml.

Thalidomide was approved for the treatment of relapsed or refractory MM as an orphan drug in Japan. Clinical background of MM patient is listed in Table 2. Although an endogenous peak (15.3 min) appeared between S-thalidomide and R-thalidomide, neither of the thalidomide enantiomers was obstructed by any endogenous peak (Fig. 3). In clinical

Table 1. Method Validation Data for Thalidomide Enantiomers in Human Whole Blood

<table>
<thead>
<tr>
<th>Spiked concentrations of thalidomide (μg/ml)</th>
<th>Mean (n=4–5) as enantiomer (μg/ml)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S-Tha</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.024</td>
<td>0.026</td>
</tr>
<tr>
<td>0.1</td>
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<td>0.049</td>
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<td>0.491</td>
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<tr>
<td>5.0</td>
<td>2.501</td>
<td>2.502</td>
</tr>
</tbody>
</table>

S-Tha, (–)-(S)-thalidomide; R-Tha, (+)-(R)-thalidomide; C.V., coefficient of variation.

Fig. 2. Chromatograms Showing (A) 1.0 μg/ml (+)-(R)-Thalidomide (R-Tha) in Methanol, (B) the Separation of 2 μg/ml racemi-Thalidomide

In clinical...
practice, patients usually take many kinds of drugs at the same time. Actually, this patient takes a lot of drugs with thalidomide (Table 3). However, we found no interference of these drugs and thalidomide on the chromatogram. In addition, an internal standard is not necessary because our technique for determination of both thalidomide enantiomers with only deproteinization has never required organic solvent extraction.

To compare between our direct deproteinization procedure and previously procedure of Murphy-Poulton et al. with liquid–liquid extraction, our method was handy and useful. The procedure for sample preparation reported by Murphy-Poulton et al. requires centrifugation, acidification, extraction, centrifugation, drying, dissolution, vortex mixing and centrifugation. On the other hand, our direct deproteinization method only needs acidification, vortex mixing and centrifugation. A comparison of the procedures for sample preparation using organic solvent extraction and using deproteinization is shown in Charts 1A, B. Only 30 min is needed for sample preparation by our procedure. In addition, only 100 µl of whole blood is sufficient to quantify both thalidomide enantiomers simultaneously by our method. In other methods, a large volume of whole blood or serum is needed for determination or quantification.

In summary, we succeeded in developing and optimizing a simple and reliable HPLC method for simultaneous determination of (+)-(R)-thalidomide and (−)-(S)-thalidomide. Complete separation of the thalidomide enantiomers and the endogenous peak achieved by using mobile phase consisting of 10% acetonitrile, 70% methanol and 20% citrate buffer (pH 3) and CHIRALpak AD-RH. The newly developed method requires only 100 µl of whole blood. The quantification limit of thalidomide as an enantiomer is 0.025 µg/ml. This HPLC determination method for both enantiomers of thalidomide is thought to be very useful for thalidomide studies.
REFERENCES

2) Lenz W., Lancet, 1, 45 (1962).