Technical Note

Simple Method for Determining Mitotane Concentration in Human Blood Plasma

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Abstract
We developed a simple method for determining mitotane concentration in a patient’s plasma using a conventional HPLC instrument equipped with an ultraviolet−visible photometer and a stainless steel column packed with octadecylsilanized silica gel. The method was validated in terms of specificity, linearity, precision, accuracy, quantitation limit, and stability of test solutions in the concentration range of 0.2 to 40 µg/mL of mitotane in plasma samples.

Keywords : mitotane, Opeprim, plasma concentration, dichlorodiphenyldichloroethane, HPLC, validation

Introduction
Mitotane \((\text{o,p}^−\text{DDD})\) is a derivative of the insecticide dichlorodiphenyltrichloroethane that specifically inhibits tumor cells of the adrenal cortex and its production of hormones. The encapsulated formulation of mitotane is known as Opeprim (500 mg capsules) and is used to treat adrenocortical tumors \([1−4]\); it causes central nervous system damage but no bone marrow depression. In the recommended treatment schedule, the patient begins at 1 to 2 capsules of Opeprim, 3 times per day, and the dose is increased incrementally up to the effective dose. The therapeutic plasma concentration range of mitotane in patient was reported as 14−20 µg/mL \([5]\). If severe side effects appear, the dose should be reduced until the maximum tolerated dose is achieved, and the plasma concentration of mitotane should be immediately determined \([6]\). The use of gas chromatography for determining the plasma concentration of mitotane has been reported \([5−7]\). However, a conventional high−performance liquid chromatography (HPLC) system is commonly used in hospitals to determine plasma concentrations of mitotane. The objective of this study was to develop and validate a simple assay that can be used with a conventional HPLC at any hospital to determine plasma mitotane concentration.

Materials
Our conventional HPLC system was composed of a Shimadzu SCL−10Avp system controller, an LC−10ADvp solvent delivery unit, an SIL−10A autosampler, a CTO−10ACvp column oven, a DGU−14A online degasser, an SPD−10AVvp ultraviolet−visible absorption detector, and CLASS−VP data−processing software on a personal computer. A stainless steel column packed with octadecysilanized silica gel (Inertsil ODS−3, 4.6 mm inside diameter, 25 cm length, 5 µm particle diameter) was purchased from GL Sciences, Inc.

Several lots of standard human plasma with added

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{Chemical Structure of Mitotane}
\end{figure}
anticoagulant (sodium heparin) were supplied by Cosmo Bio Co. and were mixed for preventing lot-to-lot variation. The mixed standard plasma was stored under freezing conditions (below −20°C) in a Sanyo medical freezer MDF-U536.

Fluka 2,4–DDD (cat. no. 35485, PESTANAL, analytical standard, not less than 99%) was used as the standard mitotane substance for the determination. Wako p,p–DDD standard (cat. no. 046–27101, for determining residual pesticides) was used as an internal standard (IS).

An Ultrafree–MC centrifugal filter device (pore size: 0.45 µm; cat. no. UFC 30 HVNB, Millipore Corp.) was used, and the sample was centrifuged with a centrifugal separator, model CR–15, supplied by Hitachi–Koki Co.

A solvent gradient of purified water and methanol was used as a mobile phase. Acetonitrile and methanol for the liquid chromatography were supplied by Kanto Chemical Co. and Wako Pure Chemical Industries. Purified water with an electrical resistance of at least 18.2 MΩ·cm was prepared with a Milli–Q Ultrapure production unit (Millipore Corp.).

Test procedure

Preparation of Standard Stock Solutions (STK−1, STK−2)

To prepare standard stock solution 1 (STK−1, 400 µg/mL), approximately 20 mg of mitotane standard, accurately weighed, was dissolved in methanol to a final volume of 50 mL. STK−1 (1 mL) was diluted with methanol to a final volume of 20 mL, and the resulting solution was designated as standard stock solution 2 (STK−2, 20 µg/mL). STK−1 can be stored at 5 ± 3°C for 4 weeks, but STK−2 should not be stored.

Preparation of Standard Solutions (SD solutions)

Methanol was added to 8, 4, 2, and 1 mL of STK−1 to make a final volume of 20 mL for each solution, and these solutions were designated as SD–160 (160 µg/mL), SD–80 (80 µg/mL), SD–40 (40 µg/mL), and SD–20 (20 µg/mL), respectively. Methanol was added to 8, 4, and 2 mL of STK−2 to a final volume of 20 mL for each solution, and these solutions were designated as SD–8 (8 µg/mL), SD–4 (4 µg/mL), and SD–2 (2 µg/mL), respectively. SD–0.8 (0.8 µg/mL) was prepared from 2 mL of STK−2 and enough methanol to bring the final volume to 50 mL.

Preparation of Internal Standard Solution (IS Solution)

Approximately 10 mg of p,p–DDD standard, accurately weighed, was dissolved in methanol to a final volume of 50 mL (IS stock solution: 200 µg/mL). This solution can be stored at 5 ± 3°C for 4 weeks. The IS stock solution (4 mL) was combined with sufficient methanol to bring the final volume to 20 mL (IS solution: 40 µg/mL).

Preparation of Solutions for Calibration Curve and Blank Solutions

Each solution began with 200 µL of blank plasma, to which acetonitrile, SD solution (or methanol for a blank sample), and IS solution were added, in this order, according to the blending ratios listed in Table 1. This solution was vortexed and then centrifuged at 10,000 rpm for 3 min. The supernatant was transferred to the sample reservoir of a centrifugal filter device and centrifuged at 8000 rpm for 1 min. The filtrates were used as the solutions for a calibration curve (CAL solutions) and as blank solutions. The CAL solutions and blank solutions can be stored at 5°C within 48 hours.

Preparation of Sample Solution

Acetonitrile, methanol, and IS solution were added, in this order, to 200 µL of the sample plasma. Samples were then vortexed

Table 1: Volumes of Components of Calibration Curve Solutions (CAL Solutions)

<table>
<thead>
<tr>
<th>CAL Solution</th>
<th>Blank Plasma (µL)</th>
<th>Acetonitrile (µL)</th>
<th>SD Solution (µL)</th>
<th>IS Solution (µL)</th>
<th>Mitotane Concentration (µg/mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAL–40</td>
<td>200</td>
<td>300</td>
<td>50</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>CAL–20</td>
<td>200</td>
<td>300</td>
<td>50</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>CAL–10</td>
<td>200</td>
<td>300</td>
<td>50</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>CAL–5</td>
<td>200</td>
<td>300</td>
<td>50</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>CAL–2</td>
<td>200</td>
<td>300</td>
<td>50</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>CAL–1</td>
<td>200</td>
<td>300</td>
<td>50</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>CAL–0.5</td>
<td>200</td>
<td>300</td>
<td>50</td>
<td>50</td>
<td>0.5</td>
</tr>
<tr>
<td>CAL–0.2</td>
<td>200</td>
<td>300</td>
<td>50</td>
<td>50</td>
<td>0.2</td>
</tr>
<tr>
<td>IS Blank</td>
<td>200</td>
<td>300</td>
<td>50</td>
<td>Methanol</td>
<td>50</td>
</tr>
<tr>
<td>Blank</td>
<td>200</td>
<td>300</td>
<td>50</td>
<td>Methanol</td>
<td>0</td>
</tr>
</tbody>
</table>

* In a 200–µL plasma sample
and centrifuged at 10,000 rpm for 3 min. The supernatant was transferred to the sample reservoir of a centrifugal filter device and centrifuged at 8,000 rpm for 1 min. The filtrates were used as the sample solutions and can be stored at 5 °C for 48 hours.

**Determination of Plasma Mitotane Concentration**

Twenty µL of CAL solution or blank solution was injected into the HPLC instrument, which was operated under the conditions described below. For each solution, we then calculated the ratio of the peak area of mitotane to that of the IS in each chromatogram. A calibration curve was created by plotting the peak ratio as a function of the mitotane concentration.

The test was then performed with 20 µL of the sample solution, and the peak ratio of mitotane to the IS was calculated in the same manner. The plasma concentration of mitotane can thus be obtained by the calibration curve method.

**Operation Conditions for HPLC**

- **Detector:** UV–VIS photometer (wavelength: 230 nm)
- **Column:** Inertsil ODS–3 (5 µm), 4.6 mm in inside diameter, 25 cm in length
- **Guard Column:** Guard cartridge column GL Cartridge Inertsil ODS–3, 5 µm
- **Mobile Phase A:** Purified water
- **Mobile Phase B:** Methanol

Mitotane and IS are determined under an isocratic condition. After eluting both peaks, the column is washed and re-equilibrated according to the following table.

<table>
<thead>
<tr>
<th>Time after Injection (min)</th>
<th>Mobile Phase A (vol %)</th>
<th>Mobile Phase B (vol %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>21.0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>21.1</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>34.0</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>35.0</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>45.0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>55.0</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

- **Flow Rate:** 1.0 mL/min
- **Column Temperature:** Constant temperature of 40 °C
- **Sampler Temperature:** Constant temperature of 5 °C
- **Duration of Measurement:** 55 min

**Analytical Validation**

**Specificity**

A total of 6 blank plasma samples (2 female, 4 male) were used to validate the method. The peaks for the IS and mitotane were observed in the chromatogram at 19 min and 21 min, respectively (Fig. 2). No peak appeared in the chromatogram of the blank sample at the retention time of mitotane. No difference was observed between plasma from males and females.

**Linearity and Range**

A plot of the mitotane concentrations against the ratios of the mitotane peak area to the IS peak area (8 concentrations) was linear, and correlation coefficients of 0.9998 to 0.9999 were obtained from the results of triple measurements in the concentration range of 0.2 to 40 µg/mL (Fig. 3).

**Precision and Accuracy**

The relative standard deviations obtained from 5 repeated measurements at plasma mitotane concentrations of 0.2, 0.5, 5,
and 20 µg/mL ranged between 1.2 and 2.7 %, with accuracies between –0.7 and 2.4 %. The relative standard deviations of the 3–day precision test obtained from 3 repeated measurements at plasma mitotane concentrations of 0.5, 5, and 20 µg/mL ranged between 1.7 and 2.7 %, with accuracies between –2.3 and 3.4 %.

Recovery Rate
The recovery rates of mitotane and the IS at the concentrations of both 2 and 20 µg/mL (in triplicate) were greater than 97.4 % for mitotane and greater than 98.7 % for the internal standard.

Quantitation Limit
The quantitation limit for mitotane was calculated to be 0.0013 µg for each injection as the lower limit of quantitation (LLOQ) based on the lowest spiking concentration of the analyte in Linearity and Range (corresponding to 0.2 µg/mL of plasma concentration). The relative standard deviation of triple measurements at the concentration was less than 10 %.

Stability Test of Sample and Solution
The plasma samples spiked with mitotane (0.5 and 20 µg/mL) were stable for 4 weeks at –20 °C, and the residual ratios of mitotane in the plasma samples (triplicate runs) were greater than 94.8 % at the 4–week sampling point. When the freeze–thaw cycle was repeated twice, the residual ratios of mitotane in the plasma samples (triplicate runs) were between 93.6 and 110.6 %. STK–1 was stable for 4 weeks at 5 °C, and the residual ratios of mitotane (triplicate runs) were between 99.9 and 102.4 % at the 48–hour sampling point.

Conclusion
The simple assay method for determining mitotane concentration in plasma was developed by using a conventional HPLC system equipped with an ultraviolet–visible photometer and a stainless steel column packed with octadecylsilanized silica gel. The method was validated in terms of specificity, linearity, precision, accuracy, quantitation limit, and stability of test solutions in the concentration range of 0.2 to 40 µg/mL of mitotane in plasma samples.

References