LC–MS for in Vitro Determination of a Novel Epothilone D Derivative (Epo D 7-HD) in Human Plasma

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Abstract

A novel liquid chromatography–mass spectrometry method has been established for the determination of a newly developed anti-cancer agent epothilone D derivative (Epo D 7-HD) in human plasma. The plasma sample was prepared by liquid–liquid extraction with methanol. Epo D 7-HD was quantitated on a C₁₈ column with acetonitrile-methanol (80/20, v/v) as mobile phase using LC–MS operating in positive atmospheric pressure chemical ionization with a total run time of 6.0 min. For Epo D 7-HD at the concentrations of 1, 4 and 10 μg mL⁻¹ in human plasma, the absolute extraction recoveries were 83.05, 82.37 and 80.09%, respectively. The limit of detection and quantification of Epo D 7-HD was 20 and 100 ng mL⁻¹, respectively. The linear quantification range of the method was 1–20 μg mL⁻¹ in human plasma with linear correlation coefficients greater than 0.999. The intra- and inter-day accuracy for Epo D 7-HD at 1, 4 and 10 μg mL⁻¹ levels in human plasma fell in the ranges of 95.61–101.71% and 95.28–99.80%, and the intra- and inter-day precision were in the ranges of 5.59–8.03% and 7.82–11.39%, respectively. This assay was applied to the determination of half-life of Epo D 7-HD in human plasma.

Keywords

Column liquid chromatography–mass spectrometry
Human plasma
Epothilone D derivative

Introduction

Microtubule inhibitors are an important class of anticancer drugs. Recently, epothilones, a novel secondary metabolites produced by Sorangium cellulosum with a paclitaxel-like mechanism of action have been isolated and identified [1]. Epothilones exhibit excellent promise as a new lead in cancer chemotherapy, especially where conventional treatments are ineffective [2]. The advantages of epothilones over paclitaxel and docetaxel include their increased water solubility, their more rapid action in vitro, and their effectiveness against tumor cells which exhibit multidrug resistance [2, 3]. Further, comparing with paclitaxel and docetaxel, the structures of epothilones are less complex and total chemical syntheses of several epothilones have been achieved [4, 5], one of them is epothilone D. Epothilone D demonstrates a promising therapeutic range with good activity against sensitive and multidrug-resistant tumors, and has a wide therapeutic window compared to...
epothilone B, the first investigated compound of this group [6]. In addition, epothilone D is being evaluated in human clinical trials [7].

In our laboratory, a novel epothilone D derivative (Epo D 7-HD) has been synthesized more recently and showed highly potent activities against various paclitaxel-resistant cancer cell lines at low nanomolar concentrations in the preliminary studies. The in vitro and in vivo study in our laboratory urged us to establish an analytical method for this promising drug.

In the present investigation, a rapid, simple, and sensitive liquid chromatography–mass spectrometry (LC–MS) method for the quantification of Epo D 7-HD in human plasma has been developed and validated, and further was applied to the determination of half-life of Epo D 7-HD in human plasma. Since good results in terms of precision and accuracy were achieved, no internal standard was used in this study.

**Experimental**

**Materials**

Epo D 7-HD (99.5% purity) was synthesized by the Institute of Material Medica and Department of Pharmaceutics, School of Pharmacy, Third Military Medical University, Chongqing, China. The construction of calibration curves were prepared by spiking 20 μL working solutions of Epo D 7-HD to 180 μL human plasma to achieve the concentration of 1, 2, 4, 10 and 20 μg mL⁻¹. Liquid Chromatographic (LC) grade acetonitrile and methanol were supplied by Fisher (Fair Lawn, NJ, USA).

**Liquid Chromatography–Mass Spectrometry**

The LC system consisted of a Waters Alliance 2695 Separation Module with a 996 PDA detector. LC was performed at room temperature using an XTerra MS C₁₈ column (5 μm particles, 20 × 3.0 mm), with acetonitrile–methanol (80/20, v/v) as mobile phase at a flow rate of 0.3 mL min⁻¹. 20 μL of the samples were injected and the run time was 6.0 min.

Masses were acquired on a Waters Micromass ZQ Mass Spectrometer using positive atmospheric pressure chemical ionization (APCI). Data acquisition and analyses were carried out using MassLynx version 4.0 software. Nitrogen was used as nebulizing gas (30 l h⁻¹) and desolvation gas (400 l h⁻¹) with desolvation temperature at 400 °C. The source temperature was set at 120 °C. The corona voltage was set at 4.0 μA, and cone voltage at 50 V. Full scan spectra was acquired over the m/z range of 300-900. Selected ion monitoring (SIM) mode was used for Epo D 7-HD quantitation at m/z 731 with the dwell time of 0.5 s.

**Sample Preparation**

Stock solutions of Epo D 7-HD (200 μg mL⁻¹) were prepared by dissolving accurately weighed amounts in methanol and stored at −20 °C. Working standard solutions of Epo D 7-HD were prepared in methanol at concentrations ranging from 1 ng mL⁻¹ to 100 μg mL⁻¹ by diluting the stock solution. After storage at −20 °C, stock solution and working solutions were found to be stable for at least 4 weeks.

Human plasma samples for the construction of calibration curves were prepared by spiking 20 μL working solutions of Epo D 7-HD to 180 μL human plasma to achieve the concentration of 1, 2, 4, 10 and 20 μg mL⁻¹. The resultant plasma solutions were used to evaluate the linearity of Epo D 7-HD in human plasma. Quality control (QC) samples in human plasma samples (5 injections × 3 days) were prepared in the same way at concentrations of 1, 4, and 10 μg mL⁻¹ within the range of calibration standards and were assayed each day when samples were analyzed.

**Method Validation**

The method was evaluated for specificity, accuracy, precision, stability and extraction efficiency. Frozen stability, freeze/thaw stability and extraction efficiencies were determined.

**Application of the Method**

The assay method described here was applied to study the half-life of Epo D 7-HD in human plasma. 200 μL of Epo D 7-HD stock solution were added to 3.8 mL of human plasma preincubated for 15 min (37 °C) to give a final concentration of 10 μg mL⁻¹, and incubated at 37 °C in a shaker water bath at 75 shakes min⁻¹. At the designated time, 200 μL of samples were withdrawn to a 1.5 mL microcentrifuge tube, and stored at −20 °C until analysis.

**Results and Discussion**

**Mass Spectra Analysis**

Epo D 7-HD could be detected under positive electrospray ionization (ESI⁺) mode and APCI⁺, however, APCI⁺ has higher sensitivity than ESI⁺. The full scan mass spectra of Epo D 7-HD after injection in methanol are presented in Fig. 1. The predominant protonated molecules were [M + H]⁺ m/z 731 and [M + Na]⁺ m/z 753. The mass spectrometric parameters were optimized to obtain the higher signal for the selected ions 731. The method was fully validated using ion 731 Epo D 7-HD.

**Assay Specificity**

Assay specificity was evaluated in human plasma. Chromatograms were
obtained from blank plasma at m/z 731 of Epo D 7-HD. The observed retention time of Epo D 7-HD was about 2.9 min. No significant endogenous peaks interfered with quantification of Epo D 7-HD at or near the retention time of Epo D 7-HD in the chromatogram of human plasma.

Calibration Curve

The calibration curve was obtained by plotting peak area of Epo D 7-HD versus concentration throughout the studied concentration of 1 to 20 μg mL⁻¹. A least-squares linear regression weighted by the reciprocal of the concentration was used to generate a calibration curve with correlation coefficients > 0.999.

Limit of Detection and Quantitation

The limit of detection (LOD) was defined as the quantity of Epo D 7-HD

Table 1. Assay accuracy, precision, and extraction recovery of Epo D 7-HD in human plasma

<table>
<thead>
<tr>
<th>Concentration (μg mL⁻¹)</th>
<th>Intraday accuracy</th>
<th>Interday accuracy</th>
<th>Extraction recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>RSD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>1</td>
<td>95.61 ± 5.34</td>
<td>5.59</td>
<td>96.49 ± 9.26</td>
</tr>
<tr>
<td>4</td>
<td>98.24 ± 7.89</td>
<td>8.03</td>
<td>95.28 ± 7.45</td>
</tr>
<tr>
<td>10</td>
<td>101.71 ± 6.48</td>
<td>6.37</td>
<td>99.80 ± 11.37</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation (SD) (n = 3, unit of %)
in the plasma after the sample clean-up corresponding to three times the baseline noise. Similarly, the limit of quantitation (LOQ) was defined as the signal-to-noise ratio of Epo D 7-HD > 10. Six injections were tested for the LOD and LOQ detection, respectively. The LOD and LOQ of Epo D 7-HD was 20 and 100 ng mL$^{-1}$, respectively.

**Accuracy and Precision**

The accuracy was determined by injecting three QC samples on different days. The accuracy was expressed as percentage value (% accuracy = [measured concentration/nominal concentration] × 100%). The precision was presented as percentage relative standard deviation (%RSD). The intra- and inter-day accuracy for Epo D 7-HD at 1, 4 and 10 µg mL$^{-1}$ levels in human plasma fell in the ranges of 95.61–101.71% and 95.28–99.80%, and the intra- and inter-day precision were in the ranges of 5.59–8.03% and 7.82–11.39%, respectively (Table 1).

**Stability and Extraction Efficiency**

Frozen stability and freeze-and-thaw stability were tested on QC plasma samples. QC plasma samples were frozen at −20 °C until extraction on day 1, 3, 7, 14, and 21 and then analyzed by a newly constructed calibration curve. The analytes were considered stable in the different conditions when a deviation of less than ±15% from the actual value was obtained. The results showed that the plasma samples could be stored at −20 °C for at least 14 days without any indication of degradation.

Freeze-and-thaw stability was performed with QC plasma samples at concentrations of 1, 4 and 10 µg mL$^{-1}$. QC samples were stored at −20 °C for 24 h. Aliquots were thawed unassisted at room temperature. Three aliquots for each sample were tested. When completely thawed, the samples were refrozen for approximately 24 h at −20 °C. These freeze–thaw samples were analyzed along with QC’s in order to observe whether there is any variation due to thawing of the samples. The stability data was used to supporting request for repeat analysis. The results showed that Epo D 7-HD was unstable in plasma samples through five freeze-and-thaw cycles and approximate 20% were degraded after five freeze-and-thaw cycles.

The extraction recovery was measured in QC plasma samples at concentrations of 1, 4, and 10 µg mL$^{-1}$. For recovery controls, blank plasma was well extracted. The absolute extraction recoveries of Epo D 7-HD at concentration levels of 1, 4 and 10 µg mL$^{-1}$ were 83.05, 82.37 and 80.09%, respectively (Table 1).

**Method Application**

The method was applied to investigate the half-life of Epo D 7-HD at a concentration of 10 µg mL$^{-1}$ in human plasma. The concentration–time profiles of Epo D 7-HD in human plasma was shown in Fig. 2. The half-life of Epo D 7-HD in human plasma was 22.51 h.

**Conclusion**

A rapid, simple, and sensitive LC–MS method for the quantitation of Epo D 7-HD in human plasma has been developed and validated. The assay percentage biases and RSDs were less than 12% and had a high sensitivity with the LOQ as low as 100 ng mL$^{-1}$. The method was successfully used to quantify Epo D 7-HD in human plasma, and also determine the half-life of this compound in human plasma. This method was also suitable for the pharmacology and animal pharmacokinetics of Epo D 7-HD due to the high sensitivity and specification.

**References**