Journal of Chromatography B, 877 (2009) 3748–3752

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Simultaneous determination of epothilone D and its hydrolytic metabolite in human plasma by high performance liquid chromatography–tandem mass spectrometry for pharmacokinetic studies

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ARTICLE INFO

Article history:
Received 8 January 2009
Accepted 26 August 2009
Available online 6 September 2009

Keywords:
Epothilone D
Hydrolytic metabolite
HPLC–MS/MS
Pharmacokinetics

ABSTRACT

A sensitive, simple and rapid high performance liquid chromatographic–tandem mass spectrometric (HPLC–MS/MS) method was developed for simultaneous quantitation of epothilone D and its major metabolite, the hydrolytic metabolite, epothilone C as internal standard in human plasma. Plasma samples were precipitated with acetonitrile, the analysis used a Venusil ASB C_{18} analytical column. A tandem mass spectrometer equipped with electrospray ionization source was used as detector and operated in the positive-ion mode. Selected reaction monitoring (SRM) using the precursor to product ion pairs of m/z 492.3 → 304.1 (epothilone D), m/z 510.3 → 492.3 (metabolite), m/z 478.3 → 290.1 (internal standard) was used for quantification. The analytical method was validated in terms of specificity, precision, accuracy, extraction recovery, stability, matrix effect and dilution effect. The linear calibration curves of epothilone D and metabolite were obtained over the concentration range of 0.2–1000 ng/ml and 5.0–1000 ng/ml, respectively. Lower limits of quantification (LLOQ) of epothilone D and metabolite were 0.2 ng/ml and 5.0 ng/ml, respectively. Due to its high sensitivity, specificity and simplicity, the method could be used for pharmacokinetic studies of both epothilone D and its hydrolytic metabolite.

1. Introduction

The epothilones are a new class of 16-membered macrolides, which are currently under evaluation for antitumor potential both in vitro and in vivo [1–6]. They share a similar cellular mechanism of action to taxol, both the epothilones and taxol exert their biological effects by stabilizing microtubule assemblies, thus leading to the arrest of cell division and eventual cell death [1]. More importantly, the epothilones inhibit the growth of cancer cells that overexpress the P-glycoprotein efflux pump and are resistant to various other chemotherapeutic agents through the MDR-mediated drug resistance mechanism [7,8]. In addition, the epothilones are also more water-soluble and more easily available through chemical synthesis than taxol [1]. Epothilone D is equivalent to other epothilones and more potent than paclitaxel [9], although its antiproliferative activity is somewhat less than that of other epothilones. In contrast to paclitaxel, EpoD demonstrates efficacy in a wide variety of tumors, including those resistant to paclitaxel, vinblastine, and doxorubicin [5,6]. For its further study, it is necessary to develop an analytical method for the determination of such a compound, especially in biological fluids. Some analytical methods were reported for epothilone D and epothilone D analog, high performance liquid chromatography (HPLC) [1] and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) [10]. And a few papers were reported preclinical pharmacology of epothilone D. In order to satisfy the urgent request of clinical study, in this research, we develop a LC/MS/MS method that employed a simple acetonitrile precipitation procedure for analyte recovery and that allowed sensitive and rapid quantitation of epothilone D and its hydrolytic metabolite in human plasma simultaneously. The method is applied to the pharmacokinetic study of epothilone D in clinical investigation.

2. Experimental

2.1. Reagents and materials

Epothilone D (purity 99.3%) (Fig. 1a), hydrolytic metabolite (purity 97.9%) (Fig. 1b) and epothilone C as internal standard (purity 99.7%) (Fig. 1c), were graciously provided by Beijing Institute of Radiation Medicine (Beijing, China). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (HPLC grade) was purchased from Sigma–Aldrich (St. Louis, MO). Distilled water was purified using a SZ-93A auto-

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1570-0232/$ – see front matter.
doi:10.1016/j.jchromb.2009.08.052

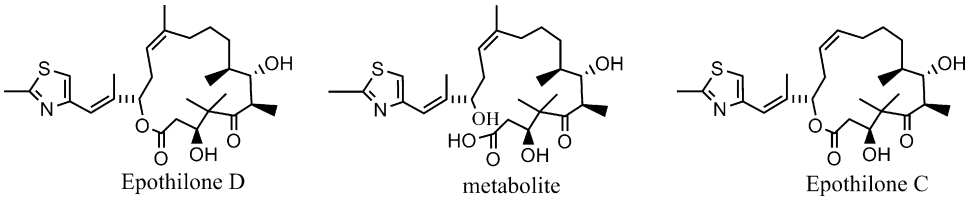

Fig. 1. Structure of epothilone D (a), hydrolytic metabolite of epothilone D (b) and epothilone C (internal standard) (c).

double distillation apparatus (YaRong Corp., Shanghai, China). Argon (≥99.999%) and liquid nitrogen (≥99.999%) were purchased from the Beijing HaiKeYuanChang Co., Ltd (Beijing, China). Blank human plasma was obtained from the Beijing Red Cross Blood Center (Beijing, China).

2.2. HPLC conditions

The HPLC system consisted of a Finnigan Surveyor Autosampler Plus, and a Finnigan Surveyor MS Pump Plus (Thermo Electron Corporation, USA). The analytical column was Venusil ASB C_{18} (2.1 mm × 150 mm, 5 μm, Agela Technologies Inc., USA). The mobile phases were as follows: mobile phase solvent A was methanol (contain 0.1% formic acid v/v) and mobile phase solvent B was distilled water (contain 0.1% formic acid v/v). The initial mobile phase composition of 10% solvent A and 90% solvent B was maintained for 1 min. Between 1 and 6 min, the percentage of solvent A was changed to 100%. Between 6 and 7 min, the percentage of solvent A was then returned to 10%. The overall run time was 7 min. The flow rate was 0.2 ml/min and the injection volume was 20 μl.

2.3. Mass spectrometer conditions

Mass detection was carried out using a Finnigan TSQ Quantum Ultra equipped with a heated-electrospray ion source and operated by Xcalibur software Version 2.0 (Thermo Electron Corporation, USA), with positive-ion, selected reaction monitoring (SRM) mode. The settings of the mass spectrometer were as follows: spray voltage 4.5 kV; capillary temperature 300 °C. The sheath gas and auxiliary gas pressures were 23 and 16 psi, respectively. The analytes were detected by monitoring the transitions m/z 492.3 → 304.1, 510.3 → 492.3 and 478.3 → 290.1 with the collision energy 20, 28 and 12 eV for epothilone D, hydrolytic metabolite and internal standard, respectively. The ionspray interface and mass spectrometric parameters were optimized to obtain maximum sensitivity at unit resolution.

2.4. Preparation of stock and working solutions

Stock solution containing 2 mg/ml epothilone D and hydrolytic metabolite were prepared by dissolving each respective compound in methanol, and stored at −20 °C. Stock solutions were combined in order to obtain a single solution that had a final concentration of 1 mg/ml for each analyte, and then a serial dilution was made to achieve the desired serial concentrations of working solutions.

2.5. Preparation of calibration curve and quality control (QC) samples

Calibration curves and QC samples in plasma were prepared by working solutions. The final concentrations of epothilone D of calibration curve samples were 0.2, 0.5, 1, 5, 10, 50, 100, 500 and 1000 ng/ml in plasma, the hydrolytic metabolite of final concentrations of calibration curve samples were 5, 10, 50, 100, 500 and 1000 ng/ml, the final concentrations of QC samples of epothilone D and hydrolytic metabolite were 0.5, 10 and 500 ng/ml and 10, 50 and 500 ng/ml, respectively.

2.6. Sample preparation

To 100 μl calibration standard plasma samples, QC plasma samples, or clinical plasma samples were spiked with 10 μl of internal standard (500 ng/ml). After vortexing for 30 s, 150 μl acetonitrile was added to precipitate protein, then the samples were vortexed for 1 min and centrifuged at 14,000 rpm for 15 min, 20 μl of the supernatant was injected directly into the analytical column for immediate analysis.

2.7. Method validation [11,12]

Specificity was tested by an analysis of five different blank human plasma samples to ensure the absence of endogenous compounds with the same retention times as epothilone D, hydrolytic metabolite and internal standard. Calibration curve samples were prepared for every batch of totally three independent batches. LLOQ of epothilone D and hydrolytic metabolite in plasma were set at the concentration of the lowest non-zero calibration samples. The LLOQ of epothilone D and hydrolytic metabolite were defined as the lowest quantitative concentrations, taking into consideration a signal-to-noise ratio of 5. LLOQ was determined at the lowest concentration at which the precision (R.S.D.) and the accuracy (R.E.) were less than 20%. Intra-batch and inter-batch precision and accuracy were determined by assessing QC samples at low, medium and high concentration in three independent analysis batches. Precision and accuracy were expressed as relative standard deviation (R.S.D.) and relative error (R.E.), respectively.

The extraction recovery of epothilone D and hydrolytic metabolite in plasma were determined by calculating the peak areas of extracted low, medium and high QC samples against unextracted standards solutions at the same concentration representing 100% recovery.

Matrix effect was assessed in a similar fashion. Analyte for both of the two compounds were added to the extract of precipitated blank plasma to achieve concentrations of 0.5, 10, 500 ng/ml, and 10, 50, 500 ng/ml, respectively. These peak areas were compared with those obtained by adding the same concentration of analytes in acetonitrile.

In order to test whether it is possible to apply the described method to samples whose concentrations are higher than the last calibration point. The concentration of the sample out of the range of standard curve, they were diluted 10-fold and 100-fold with blank human plasma in order to bring concentration within the range of standard curve. Each analysis was performed six times using calibration curves and QC samples. The found concentrations were compared to the added ones.

The short-term stability of epothilone D and hydrolytic metabolite in plasma were determined by assessing replicate QC samples, which were kept at room temperature (25 °C) for 5 h. Freeze–thaw stability was studied after three cycles, the QC plasma samples were deproteined and the prepared samples were placed into the autosampler at room temperature for 12 h, long-term stability were

done by assessing QC samples stored at −20 °C for 2 months. The stock solution stability of epothilone D and hydrolytic metabolite were examined after storage at −20 °C for 2 months.

2.8. Analysis of clinical sample

The whole blood samples were obtained from six volunteers before, during infusing 30 min, 60 min, at the end of infusion, and 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 10 h, 24 h, 48 h, 72 h, 96 h after the injection of a 125 mg/m^2 dose of epothilone D. Administration method was intervenous drop infusion and the infusion lasted for 3 h. The plasma samples were separated from the whole blood samples and stored at −70 °C until analysis.

3. Results

3.1. HPLC–MS/MS method development

HPLC–MS/MS is a powerful technique, and now is widely used in biological analysis. During the method development of epothilone D, standard solution was introduced into mass spectrometer using syringe pump at the flow rate of 10 μl/min. Positive ionization was better than negative ionization. ESI parameters such as spray voltage, capillary temperature, sheath gas and auxiliary gas were optimized to obtain the strongest signal of product mass. Collision energies were optimized to get maximum response of the product ion. During the method development, protein precipitation was firstly tested, and accurate determination could be done when the extracts were injected into HPLC–MS/MS system. Finally, plasma samples were extracted by protein precipitation. The method proved to be timesaving and robust, compatible with the fast analysis possible with HPLC–MS/MS.

3.2. Specificity

Representative SRM chromatograms of blank plasma samples were shown in Fig. 2A. Representative chromatograms of the blank plasma spiked with working solutions were shown in Fig. 2B. Representative chromatograms of the clinical plasma were shown in Fig. 2C. It was demonstrated that there was no significant interference with the same retention times as the three analytes in blank plasma.

3.3. Calibration curve and LLOQ

Calibration curve of epothilone D and hydrolytic metabolite were validated over the concentration range of 0.2–1000 ng/ml and 5–1000 ng/ml, respectively. Typical equations of calibration curves were as follows: epothilone D, $Y_1 = -0.0033 + 0.0808X_1$ $R^2 = 0.9944$; hydrolytic metabolite, $Y_2 = 0.1646 + 0.0548X_2$ $R^2 = 0.9990$. The lower limit of quantitation of epothilone D and hydrolytic metabolite were set at the concentration of the lowest non-zero calibration samples, 0.2 and 5 ng/ml, respectively. Six replicate LLOQ samples were assayed in three independent batches. Intra-batch precision (R.S.D.%) of LLOQ samples were 4.8% and 5.0%, and inter-batch precision (R.S.D.%) of LLOQ samples were 3.3% and 2.6%, respectively. The accuracy (R.E.%) was 1.4% and −1.3%, respectively.

3.4. Precision and accuracy

Low, medium and high QC samples of epothilone D and hydrolytic metabolite in plasma were assayed in three independent batches with six replicate analyses of QC samples on each single assay, and the results are used to evaluate R.S.D.% for precision and R.E.% for accuracy and shown in Table 1.

Table 1
Precision and accuracy for the analysis of epothilone D and hydrolytic metabolite in human plasma ($n = 18$).

Compound	Added (ng/ml)	Mean ± S.D.	Intra-R.S.D.%	Inter-R.S.D.%	R.E.%
Epothilone D	0.5	0.55 ± 0.1	11.8	8.7	10.0
	10.0	10.44 ± 0.6	4.7	10.6	4.4
	500.0	478.32 ± 22.3	3.4	9.8	−4.3
Metabolite	10.0	8.6 ± 1	6.6	1.1	−14.4
	50.0	49.5 ± 4	5.9	12.1	−0.9
	500.0	480.1 ± 26	3.3	13.0	−4.0

3.5. Extraction recovery and matrix effect

The extraction recoveries of epothilone D and the metabolite were 80.8 ± 11.0, 88.9 ± 7.9, 84.4 ± 6.0, 91.3 ± 4.7, 90.4 ± 4.0 and 84.2 ± 4.6% at low, medium and high concentration, respectively. And no matrix effect was observed. The data are displayed in Table 2. The internal standard was processed in the same method. It resulted in a reproducible extraction recovery and also no matrix effect.

3.6. Effect of dilution

To investigate the effect of dilution of epothilone D and the hydrolytic metabolite, the concentration of epothilone D and the hydrolytic metabolite were both 5000 ng/ml. The samples were diluted 10-fold and 100-fold with blank human plasma respectively, in order to bring concentration within the range of standard curve. Each analysis was performed six times using calibration curves and QC samples. The found concentrations were reported and compared to the added ones, and the R.S.D.% was lower than 6.3%, and R.E.% between −5.5% and 1.5%.

3.7. Stability

The stability tests of the analytes were designed to cover expected conditions of handling of clinical samples. The stability of the analytes in human plasma was investigated under a variety of storage and processing conditions. Three freeze–thaw cycles and ambient temperature storage of the QC samples up to 5 h appeared to have no effect on results of quantification of epothilone D and the hydrolytic metabolite in plasma. The QC plasma samples were deproteined and the prepared samples were placed into the autosampler at room temperature for 12 h. The QC samples stored in a freezer at or below −20 °C remained stable for 2 months. When stock solution of epothilone D and the hydrolytic metabolite in methanol was stored at −20 °C for 2 months, the analytes were found to be stable. The detailed information are shown in Table 3.

Table 2
Matrix effect and extraction recovery of epothilone D and hydrolytic metabolite ($n = 15$).

Compound	Added (ng/ml)	Matrix effect		Extraction recovery (%)	
		Mean ± S.D.	R.S.D.	Mean ± S.D.	R.S.D.
Epothilone D	0.5	96.3 ± 6.4	6.7	80.8 ± 11.0	13.7
	10	100.3 ± 4.9	5.1	88.9 ± 7.9	10.2
	500	96.6 ± 2.5	2.6	84.4 ± 6.0	7.1
Metabolite	10	105.1 ± 5.6	5.3	91.3 ± 4.7	5.1
	50	105.6 ± 11.5	10.9	90.4 ± 4.0	4.4
	500	99.3 ± 6.4	6.4	84.2 ± 4.6	5.5

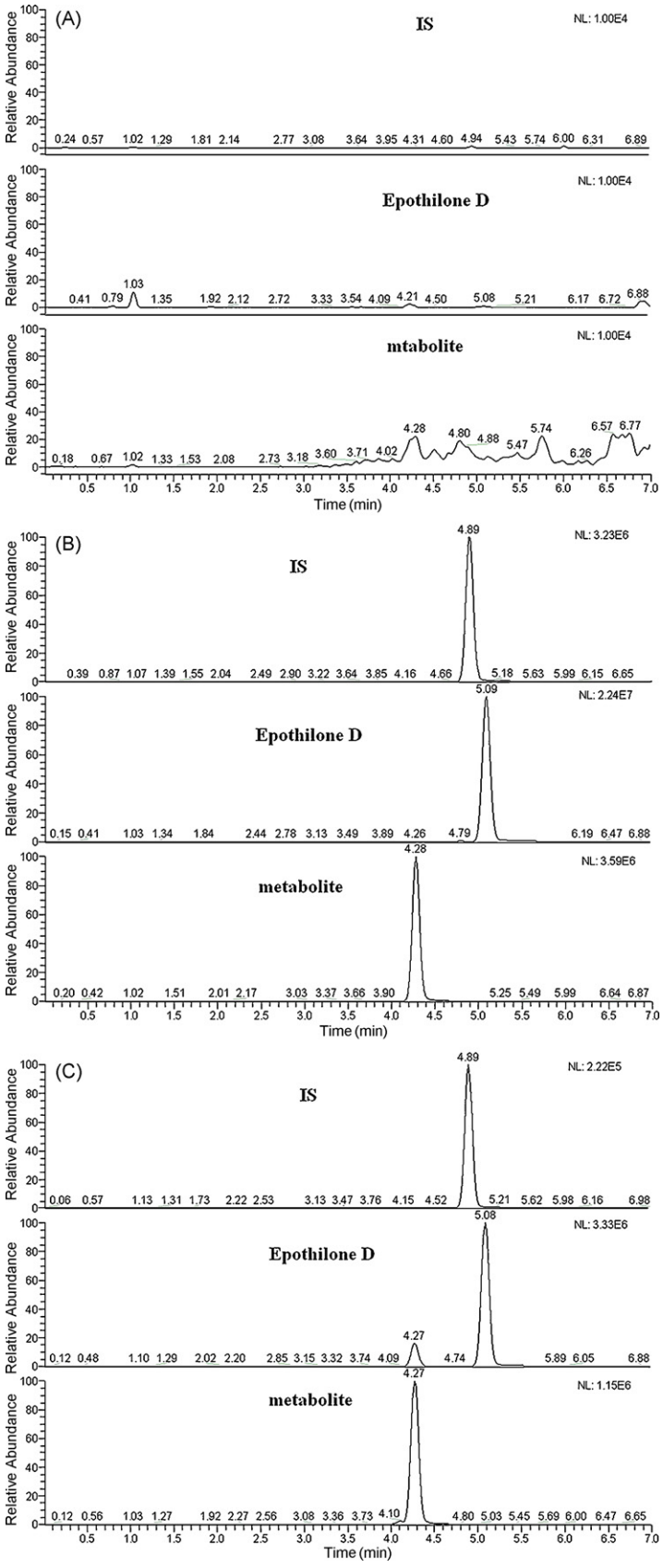

Fig. 2. SRM chromatograms of internal standard, epothilone D and metabolite. (A) Blank plasma sample; (B) blank plasma spiked with internal standard 50 ng/ml, epothilone D 50 ng/ml, hydrolytic metabolite 50 ng/ml; (C) plasma sample 1 h from volunteer after iv epothilone D infusion.

Table 3
Stability of stock solution and plasma sample of epothilone D and hydrolytic metabolite ($n = 15$).

Compound	Added (ng/ml)	Stock solution		Sample A		Sample B		Sample C		Sample D	
		Mean ± S.D.	R.E.	Mean ± S.D.	R.E.	Mean ± S.D.	R.E.	Mean ± S.D.	R.E.	Mean ± S.D.	R.E.
Epothilone D	0.5	0.5 ± 0.0	0.1	0.53 ± 0.1	6.3	0.54 ± 0.1	8.0	0.48 ± 0.1	8.0	0.55 ± 0.1	8.4
	10	10.32 ± 0.1	3.2	9.86 ± 0.2	−1.4	9.24 ± 0.5	−5.6	9.44 ± 0.6	−5.6	9.26 ± 0.2	−8.0
	500	479.95 ± 18.3	−4.0	450.52 ± 9.3	−9.9	453.27 ± 26.5	−9.4	459.50 ± 22.8	−9.4	446.65 ± 24.5	−11.9
Metabolite	10	10.5 ± 0.9	4.7	10.0 ± 0.8	0.0	10.5 ± 1.2	5.4	10.1 ± 1.2	1.1	10.6 ± 0.6	5.7
	50	52.0 ± 4.5	4.1	47.3 ± 1.7	−5.3	53.3 ± 2.5	6.7	52.2 ± 3.0	4.4	48.7 ± 5.0	−2.6
	500	513.1 ± 12.9	2.6	467.7 ± 11.3	−6.4	560.4 ± 14.7	12.1	509.7 ± 19.8	2.0	445.7 ± 20.0	−10.9

Sample A refers to the plasma samples that were kept at room temperature for 5 h; Sample B refers to the plasma samples prepared for 12 h in autosampler at room temperature; Sample C refers to the plasma samples subjected to three freeze–thaw cycles; Sample D refers to the plasma samples at −20 °C for 2 months.

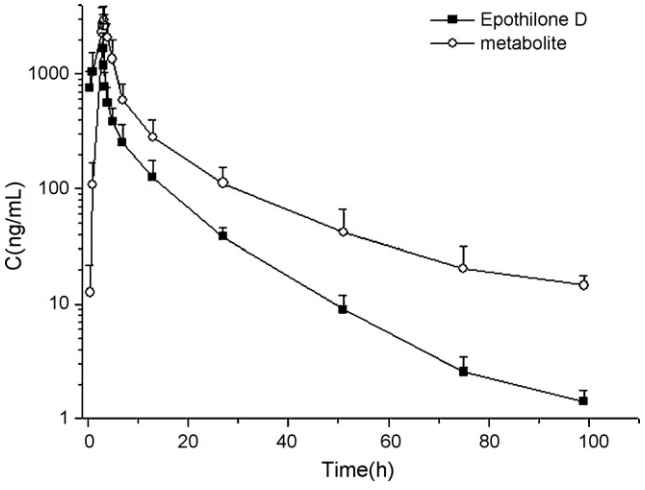

Fig. 3. Mean plasma concentration–time curves of epothilone D and its metabolite after iv infusion of epothilone D 125 mg/m^2 in volunteers ($n = 6$, mean ± S.D.).

3.8. Application to the method in pharmacokinetic studies

When applied to clinical samples, the assay proved suitable for quantitating the concentrations of epothilone D and the hydrolytic metabolite in human plasma of six volunteers treated with infusion of epothilone D infusion (125 mg/m^2). Fig. 3 presents the mean plasma concentration–time profiles of epothilone D and hydrolytic metabolite. According to the measured plasma concentrations, the main pharmacokinetic parameters were obtained as follows: for epothilone D and hydrolytic metabolite, respectively, maximum concentration (C_{max}), 2737.2 ± 1139 and 3001.6 ± 950 ng/ml; time to reach maximum concentration (T_{max}), 3.03 ± 0.0 and 3.24 ± 0.2 h; area under the curve from time 0 to 99 h (AUC_{0-99}), 9451.7 ± 2898 and 16928.2 ± 5353 μg h/L; elimination half-life ($T_{1/2}$), 15.0 ± 2 and 23.2 ± 13 h. It is evident that the data may help to establish appropriate dose and frequency of epothilone D of clinical study largely.

4. Conclusion

In order to satisfy the urgent demands for quantitating method of epothilone D and its hydrolytic metabolite in clinical study, we have established a simple, rapid and sensitive LC–MS/MS assay for simultaneous determination of epothilone D and its hydrolytic metabolite in human plasma. Some analytical methods were reported for epothilone D and epothilone D analog, to be used high performance liquid chromatography (HPLC) [1] and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) [10], respectively. In this method, the low limit of quantitation is 0.2 ng/ml which is decreased 500-fold as compared to previous methods, the sample preparation is precipitated protein with acetonitrile which is more simpler and faster than that in liquid–liquid extraction [1,10]. Only 100 μl of plasma is required for the sample pretreatment procedures, this is so important for clinical study, especially for clinical patients. This method is currently being applied in clinical pharmacokinetic study of epothilone D successfully. The ability of the assay to simultaneously quantitate epothilone D and its hydrolytic metabolite and provide information regarding the concentration versus time profile of parent compound and its metabolites, can be used to formulate the most appropriate dose and route of administration for epothilone D. An internal standard method was used successfully for quantitative analysis with high precision and accuracy because of the simple and timesaving sample pretreatment procedure. Due to its simplicity, selectivity and sensitivity, the assay is suitable for both clinical pharmacokinetic studies and routine monitoring of plasma levels of epothilone D and its metabolites.

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