Journal of Chromatography B, 879 (2011) 2018–2022

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Quantitation of paclitaxel and its two major metabolites using a liquid chromatography–electrospray ionization tandem mass spectrometry

Wei Zhang [a,b], Ginger E. Dutschman [a,b], Xin Li [b], Yung-Chi Cheng [a,b,*]

[a] Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA
[b] Yale Cancer Center, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA

ARTICLE INFO

Article history:
Received 13 January 2011
Accepted 13 May 2011
Available online 25 May 2011

Keywords:
Paclitaxel
LC–MS–MS
Metabolites
Pharmacokinetics

ABSTRACT

A sensitive and selective liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method for the determination of paclitaxel (Taxol) and its two major metabolites in human plasma has been developed. Samples were prepared after liquid–liquid extraction and analyzed on a C_{18} column interfaced with a Q-Trap tandem mass spectrometer. Positive electrospray ionization was employed as the ionization source. The mobile phase consisted of acetonitrile–water (0.05% formic acid) (65:35) at the flow rate of 0.25 mL/min. The analytes and internal standard docetaxel were both detected by use of multiple reaction monitoring mode. The method was linear in the concentration range of 0.5–500.0 ng/mL for paclitaxel, 6α-hydroxypaclitaxel and p-3′-hydroxypaclitaxel, respectively. The lower limit of quantification (LLOQ) was 0.5 ng/mL for paclitaxel, 6α-hydroxypaclitaxel and p-3′-hydroxypaclitaxel, respectively. The intra- and inter-day relative standard deviation across three validation runs over the entire concentration range was less than 8.18%. The accuracy determined at three concentrations was within ±10.8% in terms of relative error. The total run time was 7.0 min. This assay offers advantages in terms of expediency, and suitability for the analysis of paclitaxel and its metabolites in various biological fluids.

© 2011 Published by Elsevier B.V.

1. Introduction

Paclitaxel was originally isolated in the bark of the Pacific Yew Tree and is now prepared using a semi-synthetic method [1,2]. It was approved by the United States Food and Drug Administration (FDA) in 1992 and launched one year later in the USA. Paclitaxel causes cell death via microtubule stabilization and is used to treat ovarian cancer, lung cancer, head and neck cancer, bladder cancer, and AIDS-related Kaposi's sarcoma [3–5]. It can also be used together with cisplatin to treat advanced ovarian cancer and non-small cell lung cancer [6–8].

Paclitaxel is metabolized primarily in liver, and its two main metabolites are 6α-hydroxypaclitaxel (6α-OHP, with an OH group on the phenyl at C6 of the taxane ring) that was formed by human liver cytochrome P-450 (CYP) 2C8 and p-3′-hydroxypaclitaxel (C3′-OHP) was formed by CYP3A4 [9,10]. A minor di-OHP metabolite, formed from 6α-OHP was also found in human liver slices and microsomes. The metabolites of paclitaxel were less active and cytotoxic than paclitaxel *in vitro*. Now paclitaxel plus other anti-cancer drugs were widely studied in different clinical trial [11–13]. For a better usage of paclitaxel and studying the drug interaction, it is important to learn the pharmacokinetics of paclitaxel and its metabolites. These considerations underscore the potential utility of a rapid and convenient assay for the measurement of paclitaxel and its major metabolites.

Several high-performance liquid chromatography methods have been developed for the determination of paclitaxel in biological fluids. For example, Suno et al. determined the paclitaxel and its metabolites in human plasma by liquid chromatography with ultra-violet visible (UV–vis) spectrophotometry [14–16]. However those methods require the use of expensive solid phase extraction cartridges or large volumes (0.4–1.0 mL) of sample to achieve low quantitation limits of approximately 3 ng/mL. Although a number of more sensitive mass spectrophotometric methods have been developed for paclitaxel, only a few are for the determination of paclitaxel and its metabolites in plasma [17–19]. An LC/MS/MS method employing 50 μL of sample and solid phase extraction procedure reported a sensitivity of 36 ng/mL in the plasma [17]. Another report achieved a sensitivity limit of 0.1 ng/mL, yet 400 μL of human plasma and a time-consuming freezer procedure (approximately 45 min) were required [18]. Simple liquid–liquid extraction procedure and short run time can curtail test's time that is important for large sample batches.

In this paper, we described a simpler, selective and highly sensitive HPLC–MS/MS method using small volumes of plasma from humans, which allow the simultaneous quantification of paclitaxel, 6α-OHP and C3′-OHP based on a simpler liquid–liquid

* Corresponding author at: Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA.
E-mail address: wei.zhang87@yahoo.com (Y.-C. Cheng).

1570-0232/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2011.05.024

extraction. This method is fully validated for clinical studies and therefore could be the basis for further clinical studies with paclitaxel.

2. Experimental

2.1. Materials

Paclitaxel, docetaxel (internal standard, I.S.) were purchased from Sigma (St. Louis, MO, USA). 6α-Hydroxypaclitaxel and p-3′-hydroxypaclitaxel were purchased from BD Biosciences (San Jose, CA, USA). Ether and chloroform were obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA) and American Bioanalytical (Natick, MA, USA), respectively. HPLC-mass grade methanol, acetonitrile, water and 0.05% formic acid were obtained from J.T.Baker (Phillipsburg, NJ, USA).

2.2. Instrumentation

The chromatographic system used consisted of an Agilent 1200 HPLC series, including a binary pump (Model G1312B), a vacuum degasser (Model G1379B), an autosampler (Model G1367C) and a column oven (Model G1316B). The mass spectrometer was an Applied Biosystems Sciex 4000 Q-trap® mass spectrometer (Applied Biosystems Sciex, Foster, CA, USA). Data acquisition was carried out by Analyst 1.4.2® software on a DELL computer.

2.3. LC–MS conditions

The chromatographic separation was achieved on a ZORBAXSB-C_{18} column (50 mm × 2.1 mm i.d., 3.5 μm, Agilent, Palo Alto, CA, USA). The mobile phase was acetonitrile–0.05% formic acid = 65:35 (v/v) at the flow rate of 0.25 mL/min. The column temperature was maintained at 30 °C. After chromatographic separation, the mobile phase was directly introduced into the mass spectrometer via electrospray ionization (ESI) source operating in the positive mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 876.4 → m/z 308.1 for paclitaxel, m/z 892.4 → m/z 607.2 for 6α-OHP, m/z 892.4 → m/z 324.1 for C3′-OHP, m/z 830.4 → m/z 304.1 for docetaxel (internal standard, I.S.), respectively, with a dwell time of 150 ms. In order to optimize all the MS parameters, a standard solution (0.1 μg/mL) of the analyte and I.S. was infused into the mass spectrometer. Some mass spectrometer parameters were identical for all analytes. The curtain gas reached 15 psi. The ionspray voltage was set at 5500 V and the temperature at 700 °C. The nebulizer gas (GS1) and turbo gas (GS2) were 55 psi and 55 psi. The declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) were optimized for each analyte. The declustering potentials were set at 90, 90, 70 and 70 V for paclitaxel, 6α-OHP, C3′-OHP and I.S., respectively. The values of the collision energy were 40, 30, 40 and 34 V for paclitaxel, 6α-OHP, C3′-OHP and I.S., respectively. The collision cell exit potentials were 5, 5, 15 and 5 V for paclitaxel, 6α-OHP, C3′-OHP and I.S., respectively.

2.4. Sample preparation

The plasma was prepared by removing protein through a liquid–liquid extraction method. In a 1.5 mL centrifuge tube an aliquot 100 μL of human plasma was spiked with 5 μL of docetaxel solution (internal standard, 5 μg/mL). After vortexing, 800 μL of extraction solvent (ether:chloroform = 1:1) was added to the tubes and the tubes were vortex mixed for 3 min. After centrifugation at 14,000 rpm for 10 min in the cold room, then 600 μL of clear supernatant fluid was then transferred into another centrifuge tube and evaporated to dryness in a speed Vac apparatus (Speed Vac SC 110, Savant Instruments Inc., Farmingdale, NY). The dry residue was dissolved in 100 μL solution (acetonitrile:0.05% formic acid = 65:35) with vortex-mixing for 1 min. The reconstituted plasma extract was taken and centrifuged at 14,000 rpm for 10 min and then 15 μL of supernatant fluid was injected into the HPLC–MS/MS system for analysis. The prepared samples were kept in an autosampler at 4 °C until injection.

2.5. Preparation of standard and quality control samples

Stock solutions of paclitaxel, 6α-OHP, C3′-OHP were prepared in methanol at the concentration of 100 μg/mL. Stock solution of I.S. was prepared in methanol at the concentration of 100 μg/mL and diluted to 5 μg/mL with methanol. Calibration curves were prepared by spiking the appropriate standard solution in 0.1 mL of blank plasma. Effective concentrations in plasma samples were 0.5, 1, 2.5, 10, 25, 50, 100, 250, 500 ng/mL for paclitaxel, 6α-OHP and C3′-OHP. The quality control (QC) samples were separately prepared in blank plasma at the concentrations of 2.5, 25 and 250 ng/mL for paclitaxel, 6α-OHP and C3′-OHP, respectively. The spiked plasma samples (standards and quality controls) were then treated following the "Sample preparation" procedure on each analytical batch along with the unknown samples.

2.6. Method validation

Plasma samples were quantified using the ratio of the peak area of each analytes to that of I.S. as the assay parameter. Peak area ratios were plotted against analytes concentrations and standard curves were in the form of $y = A + Bx$.

To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on three separate days. The accuracy and precision were also assessed by determining QC samples at three concentration levels on three different validation days. The accuracy was expressed by (mean observed concentration − theoretical concentration)/(theoretical concentration) × 100% and the precision by relative standard deviation (RSD %).

Absolute recoveries of paclitaxel, 6α-OHP and C3′-OHP at three QC levels were determined by assaying the samples as described above and comparing the peak areas of paclitaxel, 6α-OHP C3′-OHP and I.S. with those obtained from direct injection of the compounds dissolved in the supernatant of the processed blank plasma.

2.7. Matrix effects (ME)

To evaluate the absolute matrix effect, i.e., the potential ion suppression or enhancement due to co-eluting matrix components, five different batches of blank plasma were extracted and then spiked with the analytes at three QC concentrations. The corresponding peak areas of the analytes in spiked plasma post-extraction (A) were then compared to those of the aqueous standards in mobile phase (B) at equivalent concentrations. The ratio ($A/B \times 100$) is defined as the ME. A ME value of 100% indicates that the responses for analytes in the mobile phase and in the plasma extracts were the same and that no absolute ME was observed. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression. The assessment of the relative ME was made by a direct comparison of the analytes peak area values between different lots sources of plasma. The variability in the values, expressed as RSD (%), is a measure of the relative ME for the target analytes.

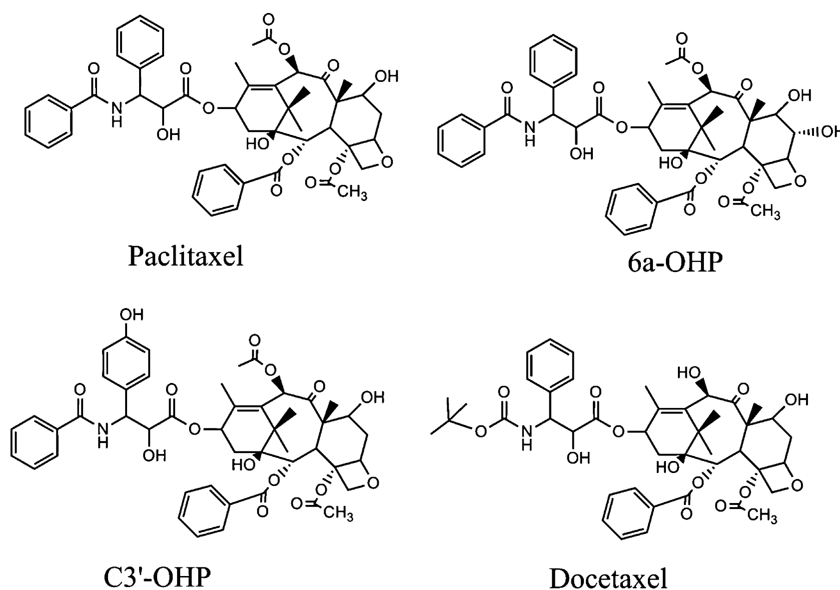

Fig. 1. The structures of paclitaxel and its metabolites.

3. Results and discussion

3.1. Mass spectrometry

Because paclitaxel, 6α-OHP, C3′-OHP and I.S. have numerous basic functional groups (Fig. 1), the positive ionization mode was initially chosen. The Q1 full scan spectra of paclitaxel and I.S. were dominated by protonated molecules $[M+Na]^+$, while the major ions of 6α-OHP and C3′-OHP were protonated molecules $[M+H]^+$. At the same time, there were no significant solvents adducting ions and fragments ions were observed (Fig. 2). In the product spectra of ions for paclitaxel, 6α-OHP, C3′-OHP and I.S., when the CID energy was increased more fragment ions were observed, while the response of parent ions lowered significantly. When the CID energy was set at 40, 30, 40 and 34 eV, respectively, the main fragment ion from paclitaxel, 6α-OHP, C3′-OHP and I.S. showed the highest MS response.

3.2. Preparation of plasma samples

Sample preparation is a critical step for accurate and reliable LC–MS/MS assays. The most widely employed biological sample preparation methodologies currently are liquid–liquid extraction (LLE), protein precipitation (PPT), and solid-phase extraction (SPE). Initially the PPT has been tried using acetonitrile, methanol and their mixture (2:1; 1:1). However the recoveries of paclitaxel, 6α-OHP and C3′-OHP could not reach the satisfaction levels at the same time. Liquid–liquid extraction was necessary and important

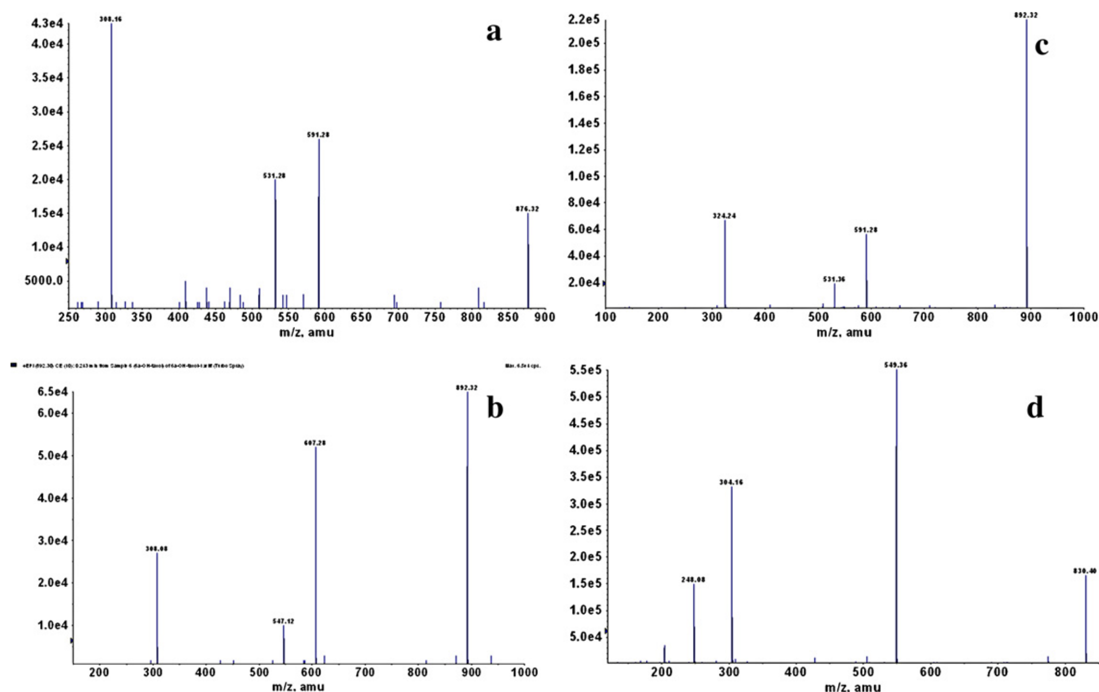

Fig. 2. Positive ion electrospray mass scan spectrum of paclitaxel and its metabolites. (A) Paclitaxel; (B) 6α-OHP; (C) C3′-OHP; and (D) I.S.

because this technique can not only purify but also concentrate the sample. Ether, ethyl acetate, dichlormethane, chloroform, ether:chloroform mixture (1:1, v/v) and ether:chloroform mixture (2:1, v/v) were all tested to do extraction and the ether:chloroform mixture (1:1, v/v) was finally adapted because of its high extraction efficiency. No alkali or acid was added to the plasma in order to accelerate the drugs' dissociation from the plasma and reduce interference since the recovery of paclitaxel was maximum at neutral condition.

3.3. Method validation

3.3.1. Specificity

The specificity tested the ability of the method to differentiate and quantitate the analyte in the presence of other endogenous constituents in the sample and to detect potential interferences. The MRM (+) chromatograms extracted from supplemented plasma are depicted in Fig. 3(B). As shown, the retention times of paclitaxel, 6α-OHP, C3′-OHP and I.S. were 4.18, 3.27, 2.00 and 4.97 min, respectively. The total HPLC–MS analysis time was 7.0 min per sample. No interference of the analytes was observed because of the high selectivity of the MRM technique. No ion suppression effects were observed under the developed sample preparation and chromatographic conditions. Fig. 3(A) shows a HPLC chromatogram for a blank plasma sample indicating no endogenous peaks at the retention times of paclitaxel and its metabolites or internal standard (docetaxel). The typical chromatogram of a plasma sample from a patient 2.0 h after an intravenous administration of paclitaxel is depicted in Fig. 3(C). The purpose of these investigations was to develop a specific and sensitive assay for the determination of paclitaxel and its metabolites. HPLC–ESI–MS/MS has several advantages for the analysis of paclitaxel. The combination of HPLC (under the isocratic conditions described) with ESI–MS/MS leads to short retention time and yields both high selectivity and sensitivity. ESI is a 'gentle' ionization technique that produces high mass-to-charge precursor ions with minimal fragmentation of the analytes.

3.3.2. Linearity of calibration curves and lower limits of quantification

Standard curves were performed in triplicate for each analyte in plasma. In all cases the regression coefficient was >0.98. Paclitaxel and its metabolites curves were linear over a range of 0.5–500 ng/mL with a weighting on $1/x^2$. Typical standard curves were $f = 0.013C_i + 0.026$ for paclitaxel, $f = 0.056C_i + 0.0011$ for 6α-OHP and $f = 0.013C_i - 9.45E^{-6}$ for C3′-OHP. Where f represents the ratios of analyte peak area to that of I.S. and C_i represents the plasma concentrations of analyte.

The LLOQ was defined as the lowest concentration on the calibration curve for which an acceptable accuracy of ±15% and a precision below 15% were obtained. The present LC–MS/MS method offered an LLOQ of 0.5 ng/mL for paclitaxel and its two major metabolites in 0.1 mL plasma sample. This is sensitive enough to investigate the pharmacokinetic behaviors of paclitaxel and its metabolites, to establish the relationship between dose and pharmacological effect in humans.

3.3.3. ME

The ME data at three QC concentrations of paclitaxel and its metabolites in five different lots of human plasma are presented in Table 1. The results showed there was no absolute ME in this study. And the variability was acceptable, with RSD values <12.7% at different concentrations of paclitaxel and its metabolites.

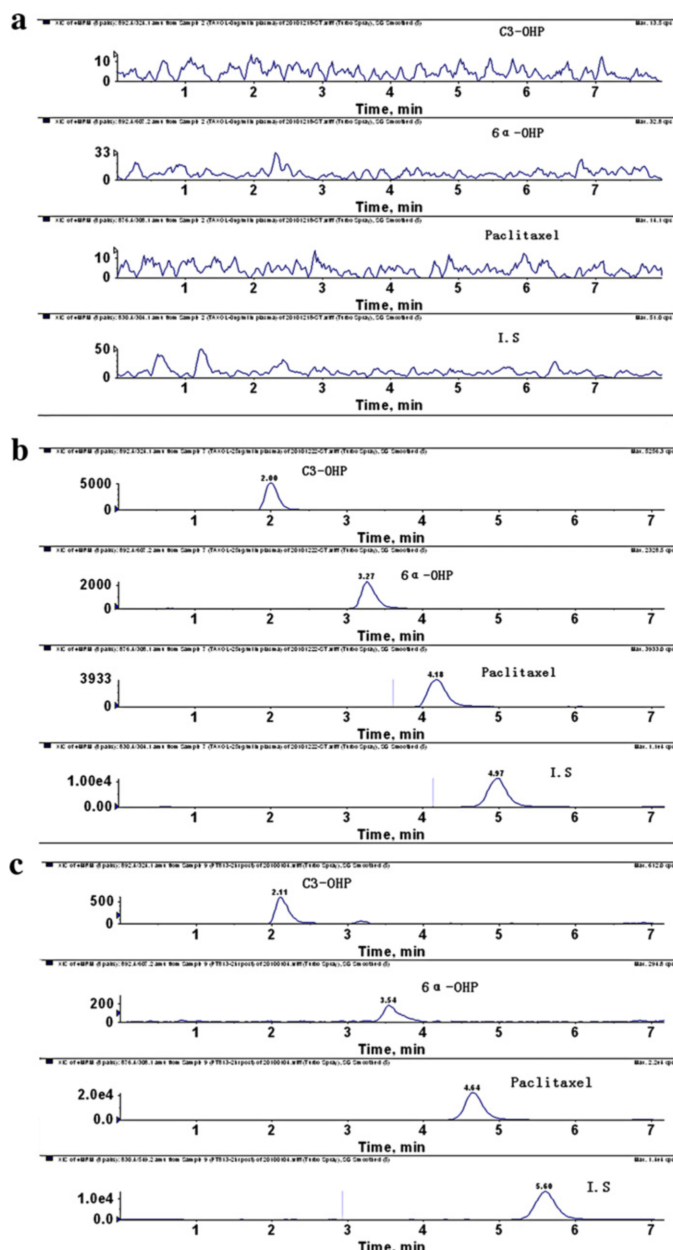

Fig. 3. Representative chromatogram of a blank human plasma and spiked plasma sample: (A) representative chromatogram of a blank human plasma; (B) representative chromatogram of a blank plasma sample spiked with paclitaxel, 6α-OHP, C3′-OHP at 25 ng/mL; and (C) representative chromatogram of a plasma sample from a patient 2.0 h after administration of paclitaxel.

Table 1
Matrix effect data for paclitaxel, 6α-OHP and C3′-OHP at 2.5, 25 and 250 ng/mL in five different lots of human plasma ($n = 5$).

	Concentration (ng/mL)	Absolute ME (mean ± SD, %)	Relative ME (%)
Paclitaxel	2.5	87.80 ± 5.1	5.9
	25.0	92.55 ± 4.1	4.5
	250.0	100.6 ± 6.5	6.5
6α-OHP	2.5	86.43 ± 8.2	9.5
	25.0	86.00 ± 6.7	7.8
	250.0	87.76 ± 7.3	8.3
C3′-OHP	2.5	92.58 ± 6.0	6.4
	25.0	107.9 ± 12.7	11.8
	250.0	90.05 ± 11.0	12.2
I.S.	250.0	102.6 ± 8.5	7.4

Table 2
Accuracy and precision for the analysis of paclitaxel, 6α-OHP and C3′-OHP in human plasma (in prestudy validation, $n = 3$ days, five replicates per day).

	Added C (ng/mL)	Found C (ng/mL)	Intra-run RSD (%)	Inter-run RSD (%)	Relative error (%)
Paclitaxel	2.5	2.53	7.67	7.56	1.2
	25.0	24.6	5.89	5.97	−1.6
	250.0	276.9	4.80	3.10	10.8
6α-OHP	2.5	2.46	6.86	8.00	−1.6
	25.0	26.6	6.30	5.79	6.4
	250.0	225.7	2.31	4.42	−9.72
C3′-OHP	2.5	2.54	6.42	8.10	1.6
	25.0	25.5	8.18	7.56	2.0
	250.0	237.5	1.32	2.75	−5.0

3.3.4. Precision and accuracy

Table 2 summarizes the intra- and inter-day precision and accuracy for paclitaxel and its metabolites evaluated by assaying the QC samples. The precision was calculated by using one-way ANOVA. In this assay, the intra-run precision was 8.18% or less, and the inter-run precision was 8.10% or less for each QC level of paclitaxel and its metabolites. The accuracy was within ±10.8%. The results above demonstrated that the values were within the acceptable range and the method was accurate and precise.

3.3.5. Recovery and stability

The recovery of paclitaxel, 6α-OHP and C3′-OHP, determined at three concentrations (2.5, 25, 250 ng/mL) were 64.65 ± 15.5%, 64.49 ± 5.4%, 69.49 ± 2.3%; 83.96 ± 13.1%, 81.21 ± 7.2%, 76.37 ± 1.9% and 73.25.65 ± 15.9%, 80.63 ± 7.5%, 79.55 ± 7.4%; ($n = 5$), respectively.

The stabilities of QC samples at three different concentrations (2.5, 25, 250 ng/mL) in the whole blood prepared according to the above-mentioned method were tested by short-term stability (4 °C) assays. Paclitaxel, 6α-OHP and C3′-OHP were stable for 24 h at different concentrations ranging from 92.8% to 115.9%.

4. Conclusions

The proposed method of analysis provided a sensitive and specific assay for determination of paclitaxel and its metabolites in human plasma. The simple liquid–liquid extraction procedure and short LC–MS/MS run time can allow a large number of samples to be analyzed. It was shown that this method is suitable for the analysis of paclitaxel and its metabolites in human plasma samples collected for pharmacokinetic, bioavailability or bioequivalence studies in humans. The results proved that the method is rapid, sensitive and highly selective.

Acknowledgements

This work is supported by NIH Grants RO1CA63477 and Yale Comprehensive Cancer Center Grants #3P30CA16359. Y-C. Cheng is a fellow of the National Foundation for Cancer Research.

References

[1] B. Ganem, R.R. Franke, J. Org. Chem. 72 (2007) 3981.
[2] H. Tabata, Curr. Drug Targets 7 (2006) 453.
[3] A. Younes, J.P. Ayoub, A. Sarris, L. North, O. Pate, P. McLaughlin, M.A. Rodriguez, J. Romaguera, F. Hagemeister, C. Bachier, A. Preti, F. Cabanillas, Ann. Oncol. 8 (Suppl. 1) (1997) 129.
[4] W.B. Derry, L. Wilson, M.A. Jordan, Cancer Res. 58 (1998) 1177.
[5] M.A. Jordan, K. Wendell, S. Gardiner, W.B. Derry, H. Copp, L. Wilson, Cancer Res. 56 (1996) 816.
[6] N.H. Oberlies, D.J. Kroll, J. Nat. Prod. 67 (2004) 129.
[7] G. Frasci, P. Comella, G. De Cataldis, L. Maiorino, F. Fiore, V. De Rosa, L. Lapenta, M. Licenziato, G. Comella, Oncology 65 (2003) 378.
[8] J. Klastersky, J.P. Sculier, Lung Cancer 12 (Suppl. 2) (1995) S117.
[9] R. Vaclavikova, P. Soucek, L. Svobodova, P. Anzenbacher, P. Simek, F.P. Guengerich, I. Gut, Drug Metab. Dispos. 32 (2004) 666.
[10] E.K. Rowinsky, M. Wright, B. Monsarrat, R.C. Donehower, Ann. Oncol. 5 (Suppl. 6) (1994) S7.
[11] L. Gianni, L. Vigano, A. Locatelli, G. Capri, A. Giani, E. Tarenzi, G. Bonadonna, J. Clin. Oncol. 15 (1997) 1906.
[12] K. Miller, M. Wang, J. Gralow, M. Dickler, M. Cobleigh, E.A. Perez, T. Shenkier, D. Cella, N.E. Davidson, N. Engl. J. Med. 357 (2007) 2666.
[13] T.S. Bekaii-Saab, J. Liu, K.K. Chan, S.P. Balcerzak, P.S. Ivy, M.R. Grever, E.H. Kraut, Clin. Cancer Res. 14 (2008) 3434.
[14] M. Suno, T. Ono, S. Iida, N. Umetsu, K. Ohtaki, T. Yamada, T. Awaya, M. Satomi, Y. Tasaki, K. Shimizu, K. Matsubara, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 860 (2007) 141.
[15] H. Rosing, V. Lustig, F.P. Koopman, W.W. ten Bokkel Huinink, J.H. Beijnen, J. Chromatogr. B 696 (1) (1997) 89.
[16] J.G. Supko, R.V. Nair, M.V. Seiden, H. Lu, J. Pharm. Biomed. Anal. 21 (5) (1999) 1025.
[17] P. Guo, J. Ma, S. Li, J.M. Gallo, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 798 (2003) 79.
[18] M.S. Alexander, M.M. Kiser, T. Culley, J.R. Kern, J.W. Dolan, J.D. McChesney, J. Zygmunt, S.J. Bannister, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 785 (2003) 253.
[19] R.A. Parise, R.K. Ramanathan, W.C. Zamboni, M.J. Egorin, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 783 (2003) 231.