Journal of Chromatography B, 878 (2010) 525–537

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Liquid chromatography and tandem mass spectrometry for the quantitative determination of ixabepilone (BMS-247550, Ixempra™) in human plasma: Method validation, overcoming curve splitting issues and eliminating chromatographic interferences from degradants

Xiaohui (Sophia) Xu[a,*], Jianing Zeng[a], William Mylott[b], Mark Arnold[a], James Waltrip[b], Lisa Iacono[c], Thomas Mariannino[b], Bruce Stouffer[a]

[a] Bioanalytical Sciences, Research & Development, Bristol-Myers Squibb, Princeton, NJ 08543, USA
[b] PPD, Richmond, VA 23230, USA
[c] Discovery Medicine and Clinical Pharmacology, Research & Development, Bristol-Myers Squibb, Princeton, NJ 08543, USA

ARTICLE INFO

Article history:
Received 21 September 2009
Accepted 6 December 2009
Available online 16 December 2009

Keywords:
Ixabepilone
BMS-247550
Ixempra™
LC–MS/MS
Protein precipitation
Calibration issues
Adsorption
Degradants

ABSTRACT

A sensitive method was developed and validated for the measurement of ixabepilone (BMS-247550, Ixempra™) using a demethylated analogue of ixabepilone (BMS-212188) as an internal standard. A 0.050 mL portion of each plasma sample was extracted with 0.450 mL of acetonitrile containing the internal standard via protein precipitation. The supernatant was analyzed on a LC–MS/MS system. Chromatography was carried out on a 2.0 mm × 100 mm YMC ODS-AQ 3 μm column using an isocractic mobile phase consisting of acetonitrile:10 mM ammonium acetate, pH 5.0 (70:30, v/v) at a flow rate of 0.30 mL/min. The mass spectrometer was fitted with a TurboIonSpray® source and operated in negative ionization mode. Detection of ixabepilone and BMS-212188 were accomplished using multiple reaction monitoring (MRM) of precursor > product ion pairs of m/z 505.2 > 405.2, and 492.1 > 392.1, respectively. The assay range was 2.00–500 ng/mL and was fitted to a $1/x^2$ weighted quadratic regression model. Replicate sample analysis indicated that intra- and inter-day accuracy and precision are within ±15.0%. The recovery of ixabepilone from 0.050 mL of plasma containing 5.00 and 400 ng/mL was greater than 94%. The method was demonstrated to be sensitive, selective and robust, and was successfully used to support clinical studies. This paper also discussed approaches used for resolving a curve splitting issue observed during quantitative analysis of ixabepilone in biological matrices. Finally, to adapt the methodology to pharmacokinetics of ixabepilone after oral administration, the potential interference of chemical degradants on the determination of ixabepilone was evaluated.

Published by Elsevier B.V.

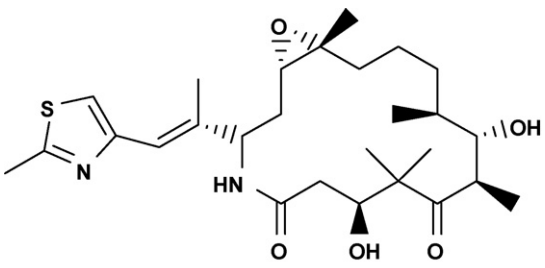
1. Introduction

Ixabepilone [1S-[1R*,3R*(E),7R*,10S*,11R*,12R*,16S*]]-7,11-dihydroxy-8,8,10,12,16-pentamethyl-3-[1-methyl-2-(2-methyl-4-thiozolyl)ethenyl]-17-oxa-4-azabicyclo[14.1.0] heptadecane-5,9-dione, BMS-247550, Fig. 1, is a semi-synthetic analogue of the natural product epothilone B, chemically modified to retain the highly favorable *in vitro* activity characteristics of natural epothilone B while improving the pharmacokinetic profile [1]. Epothilones and their analogues are a class of microtubule-stabilizing, anti-neoplastic agents, derived from the myxobacterium *Sorangium cellulosum* [1]. Ixabepilone has activity against a wide range of tumor types, including drug-resistant tumors, and has been developed for the treatment of patients with malignant tumors. Ixabepilone has demonstrated sufficient efficacy and tolerability as monotherapy or in combination with capecitabine in patients with anthracycline- and taxane-resistant metastatic breast cancer (MBC) [2–5] and has recently been approved as Ixempra™, an intravenous (IV) formulation, for use in resistant/refractory MBC.

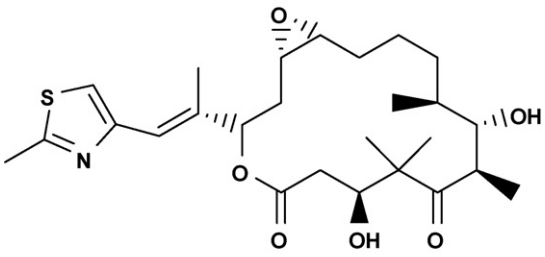
To support the clinical program, it was critical to develop a reliable bioanalytical assay to determine the concentration of ixabepilone in human plasma. High performance liquid chromatography with tandem mass spectrometry (LC–MS/MS) has been widely utilized for bioanalysis due to its selectivity and excellent sensitivity [6]. The objective was to develop and validate a simple, rapid, sensitive, and specific LC–MS/MS analytical method for the measurement of ixabepilone concentrations in human plasma. Quantitation methods were briefly described in two published

* Corresponding author at: Bristol-Myers Squibb, Mail Stop F12-01, P.O. Box 4000, Princeton, NJ 08543, USA. Tel.: +1 609 252 7271; fax: +1 609 252 3315.
E-mail address: xiaohui.xu@bms.com (X. Xu).

1570-0232/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jchromb.2009.12.014



BMS-247550
Molecular formula= $C_{27}H_{42}N_2O_5S$
Molecular Weight=506.70



BMS-212188
Molecular formula= $C_{26}H_{39}NO_6S$
Molecular Weight=493.67

Fig. 1. Structure of ixabepilone (BMS-247550) and its internal standard, BMS–212188.

Phase I clinical trials [7,8], however, this article presents the method development and validation in more detail. Ixabepilone drug substance is insoluble in water at 25 °C. The pH of a saturated solution of ixabepilone in water ranges from 6.6 to 7.1. Its aqueous solubility is unaffected by pH, which is consistent with the absence of ionizable groups. Ixabepilone is extensively metabolized [9] in human body with no major metabolites. Therefore, according to FDA's guidance for industry safety testing of drug metabolites, it was not necessary to determine the plasma level of any metabolites in pharmacokinetic studies. In this work, an off-line protein precipitation extraction, in combination with LC–MS/MS, was utilized for the determination of ixabepilone concentrations in human plasma. The method presented here was used to support a number of ixabepilone clinical studies, and has the advantage of being simple, rapid, and robust.

Curve splitting issues, where calibration curves run before and after the samples did not coincide, caused relatively higher than normal run failure rate early in the development of the bioanalytical methodology. Approaches used for understanding the cause and resolving the issues are discussed. Finally, during the development of an oral formulation of ixabepilone, it became necessary to evaluate the potential interference by isobaric chemical degradants (Fig. 2) and modify the chromatography to separate the degradants from ixabepilone.

2. Experimental

2.1. Materials

Ixabepilone (Ixempra™, BMS-247550) and BMS-212188 (Fig. 1) were obtained from Bristol-Myers Squibb (Princeton, NJ, USA). BMS-212188 is a structural analogue of ixabepilone and was used as the internal standard (IS) for this assay. The purities of ixabepilone and BMS-212188 were 99.3 and 97.3%, respectively. HPLC-grade acetonitrile was purchased from Burdick & Jackson (Morristown, NJ, USA). Acetic acid was purchased from Mallinckrodt (Hazelwood, MO, USA) and ammonium acetate was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Water was obtained from an in-house Milli-Q system (Billerica, MA, USA).

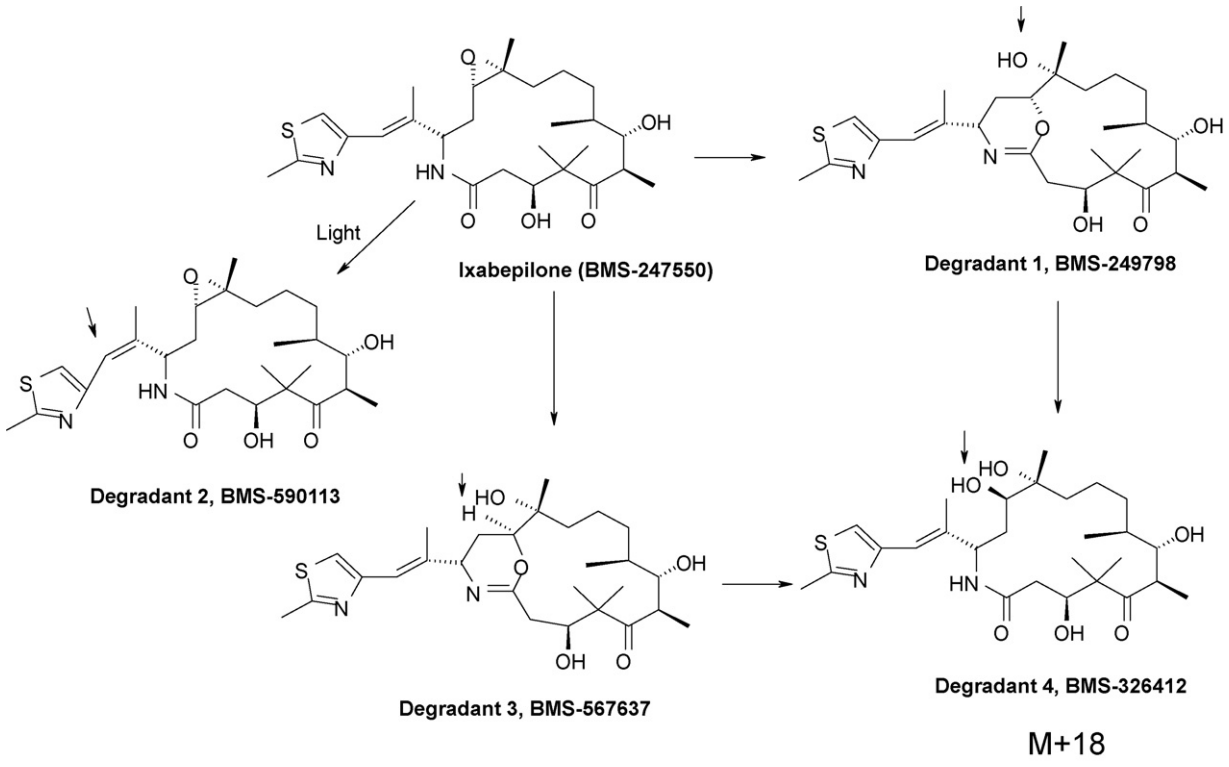


Fig. 2. Ixabepilone and its chemical degradants that may interfere with ixabepilone in SRM detection.

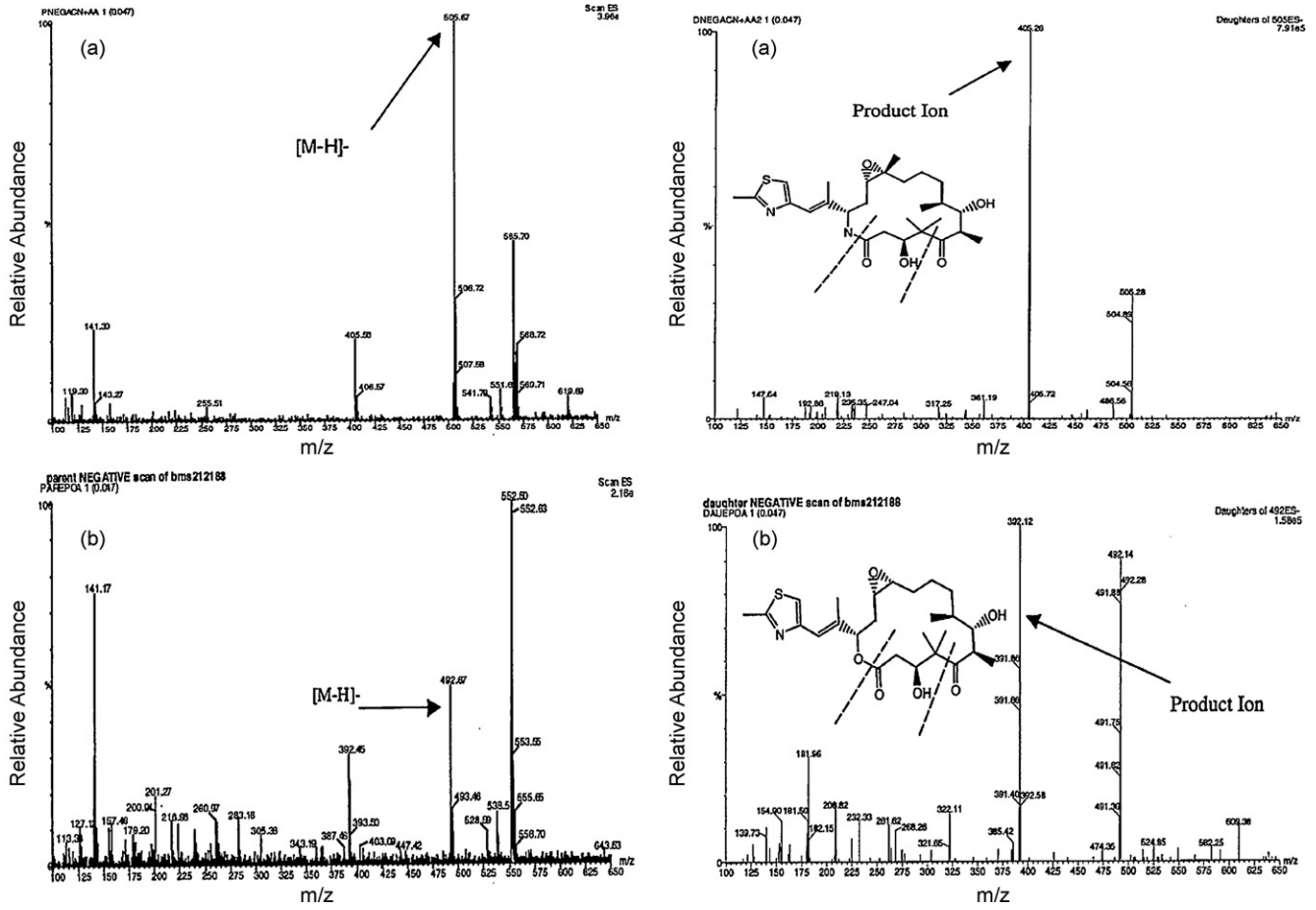


Fig. 3. (A) Precursor ion spectra $[M-H]^-$ for ixabepilone (a) and BMS-212188 (Internal Standard, b). (B) MS/MS product ion spectra for ixabepilone (a) and BMS-212188 (internal standard, b).

Control human plasma (dipotassium EDTA) was purchased from Biochemed (Winchester, VA, USA).

2.2. Instrumentation

The HPLC system consisted of a Shimadzu System Controller (Model SCL-10A Vp), Binary Pumps (Model LC 10AD Vp) and solvent degasser (Model DGU-14A) (Shimadzu Scientific Instrument, Columbia, MD, USA). A CTC Analytics PAL autosampler (CTC Analytics, Zingen, Switzerland) was used for sample injection. A Sciex API 3000 mass spectrometer (Sciex, Toronto, Canada) was used as the detector. A Tomtec robotic liquid-handling system (Hamden, CT, USA) was used for the final transfer step of the sample preparation. The data were collected and processed using Analyst software v. 1.2 (Sciex, Toronto, Canada).

2.3. Chromatographic conditions

Ixabepilone and BMS-212188 were separated on a 2.0 mm × 100 mm YMC ODS-AQ 3 μm column (YMC product number AQ12S031002WT, YMC, Milford, MA, USA). The chromatography was performed at room temperature, under isocratic conditions. The mobile phase consisted of acetonitrile:10 mM ammonium acetate, pH 5.0 (70:30, v/v), operated at 0.30 mL/min. Retention times of ixabepilone and BMS-212188 were 1.1 and 1.4 min, respectively, with a total run time was 2.5 min.

2.4. Mass spectrometer conditions

The mass spectrometer was a Sciex API-3000 equipped with a TurboIonSpray® source, operated in negative ionization mode, using multiple reaction monitoring (MRM). It is of interest to note that neither ixabepilone nor BMS-212188 have an acidic functional group that would lead to the assumption that both analytes should have poor ionization efficiency using negative ion ESI. On the contrary, both analytes demonstrate good ionization efficiency in negative ion mode. Currently, the best explanation for the good ionization efficiency lies with a process known as "wrong-way-round" ionization. The term "wrong-way-round" ionization refers to the formation of positive ions ($[M+H]^+$) under basic pH mobile phase conditions and formation of negative ions ($[M-H]^-$) under acidic pH mobile phase conditions [10,11]. Historically, the formation of ions using electrospray has been thought to occur in the liquid phase and hence to be highly dependent on the mobile phase pH. Thus "wrong-way-round" ionization conditions contradict the predicted ionization states based upon solution pH, and suggests other mechanisms of ionization are responsible for the sensitivities observed.

The mass spectrometer was operated in unit resolution mode, with Q1 and Q3 set at 0.7 Da full width at half maximum (FWHM). The optimized TurboIonSpray® and MS/MS conditions were as follows: 450 °C temperature setting, 80 psi supply N_2 gas pressure, −4200 V ion spray voltage, 8 L/min auxiliary gas flow, a setting of 12.0 for the curtain gas and a setting of 12.0 for the nebulizer gas flow. The declustering potential (DP) and entrance potential (EP) were set to −10. The focusing potential (FP) was set to −180 and

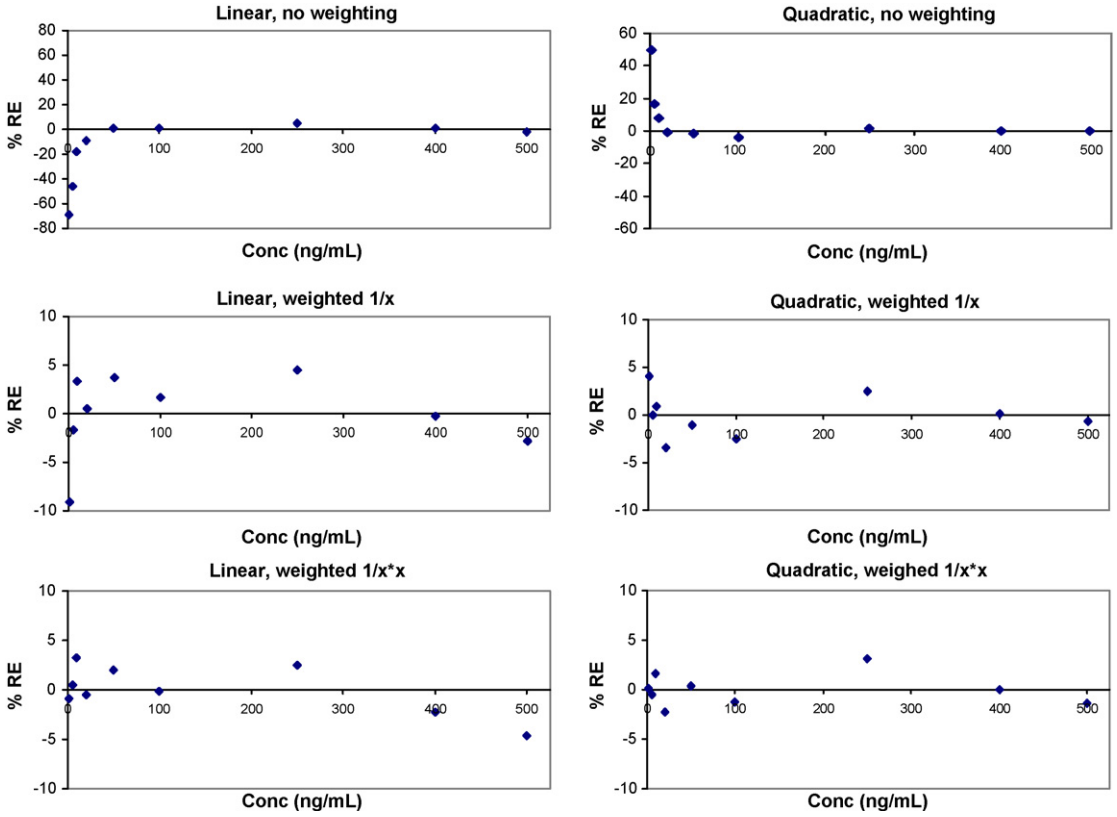


Fig. 4. Average percent relative error (%RE) versus concentration across four validation runs ($n = 8$ at each concentration).

−170 and the collision cell exit potential (CXP) was set at −13 and −11 for ixabepilone and BMS-212188, respectively. The collision energy (CE) was set at −20 and −25 eV for ixabepilone and BMS-212188, respectively. It is important to mention that system conditioning was necessary to prevent anomalies that impacted quantitation. The system conditioning will be described in Section 3.

The exact masses of ixabepilone and BMS-212188 are 506.28 and 493.25 amu, respectively. Ixabepilone gave an $[M-H]^-$ precursor ion of m/z 505.2 as the base ion. This ion was selected for collision-activated dissociation (CAD) experiments which generated a predominant product ion at m/z 405.2. The internal standard, BMS-212188, gave an $[M-H]^-$ precursor ion of m/z of 492.1. This ion was selected for CAD experiments which generated a predominant product ion at m/z 392.1. The precursor-to-product ion transitions used were 505.2 > 405.2 and 492.1 > 392.1 for ixabepilone and BMS-212188, respectively, and each had a dwell time of 200 ms. Precursor and product ion scans, 100–650 amu, for ixabepilone and BMS-212188 are shown in Fig. 3A and B, respectively. The mass assignment accuracy is known to be limited when scanning large mass ranges. Therefore, the mass assignments used for the MRM were based on precursor and product ion scans over a much narrower mass range (~10 amu).

2.5. Standards and quality controls (QC)

Prior to making calibration standards and quality controls, two independent ixabepilone stock solutions were prepared at 100 μg/mL in acetonitrile and their concentrations verified against each other (% difference ≤5%). One stock solution was designated for the use to prepare standards and the other stock solution was designated for use to prepare quality control samples. Ixabepilone stock solutions have been demonstrated to be stable for up to 306 days when prepared in acetonitrile and stored at −20 °C.

From the verified standard stock solution, an ixabepilone intermediate working solution was prepared at 2500 ng/mL in dipotassium EDTA (K_2EDTA) human plasma and discarded after use. Calibration standards were prepared fresh on the day of use, for each validation run, by serial dilution from the above intermediate working solution in human K_2EDTA plasma, at nominal ixabepilone concentrations of 2.00, 5.00, 10.0, 20.0, 50.0, 100, 250, 400 and 500 ng/mL. A nine-point calibration standard curve ranging from 2.00 to 500 ng/mL of ixabepilone in human K_2EDTA plasma was used in duplicate in each analytical run.

From the verified quality control sample stock solution, an ixabepilone intermediate working solution (2500 ng/mL) was prepared by diluting 50 μL of the 0.100 mg/mL QC stock solution with 1.95 mL human plasma containing K_2EDTA and discarded after use. Quality control pools were prepared from the 100 μg/mL stock solution and the above intermediate working solution in human K_2EDTA plasma, at nominal ixabepilone concentrations of 2.00, 5.00, 30.0, 200, 400 and 4000 ng/mL. After thorough mixing, each quality control pool was divided into smaller volumes (approximately 0.5 mL) and stored in polypropylene tubes at −70 °C.

A BMS-212188 stock solution was prepared at 100 μg/mL in acetonitrile. From the 100 μg/mL stock solution, a BMS-212188 working internal standard solution was prepared at 120 ng/mL in acetonitrile. BMS-212188 stock solutions have been demonstrated to be stable for up to 289 days when prepared in acetonitrile and stored at −20 °C.

2.6. Sample preparation

Human plasma samples were thawed at room temperature and vortex-mixed to ensure uniformity prior to transferring. A 50.0 μL

Table 1
Back-calculated concentration of individual standards from accuracy and precision runs.

Run no.	2.00 ng/mL	5.00 ng/mL	10.0 ng/mL	20.0 ng/mL	50.0 ng/mL	100 ng/mL	250 ng/mL	400 ng/mL	500 ng/mL
1	2.13	4.84	9.77	18.51	51.23	91.80	268.49	404.01	451.28
	1.85	5.25	10.92	19.51	47.12	102.33	283.90	390.36	508.40
2	1.97	4.78	10.33	19.21	49.26	96.73	247.94	402.78	496.56
	2.06	4.95	10.31	19.62	53.43	101.94	249.80	398.93	503.49
3	1.92	5.03	10.42	19.51	51.53	100.82	258.74	406.12	505.62
	2.10	4.84	9.65	19.90	48.37	100.43	256.69	390.14	481.83
4	2.08	4.76	9.73	20.09	49.94	99.03	246.82	401.46	495.26
	1.90	5.37	10.20	20.12	50.76	97.16	250.96	406.42	501.97
Mean	2.00	4.98	10.17	19.56	50.20	98.78	257.92	400.03	493.05
SD	0.11	0.23	0.43	0.53	1.97	3.49	12.68	6.50	18.78
%CV	5.27	4.55	4.24	2.70	3.93	3.53	4.92	1.63	3.81
%Deviation	0.07	−0.47	1.67	−2.21	0.41	−1.22	3.17	0.01	−1.39
n	8	8	8	8	8	8	8	8	8

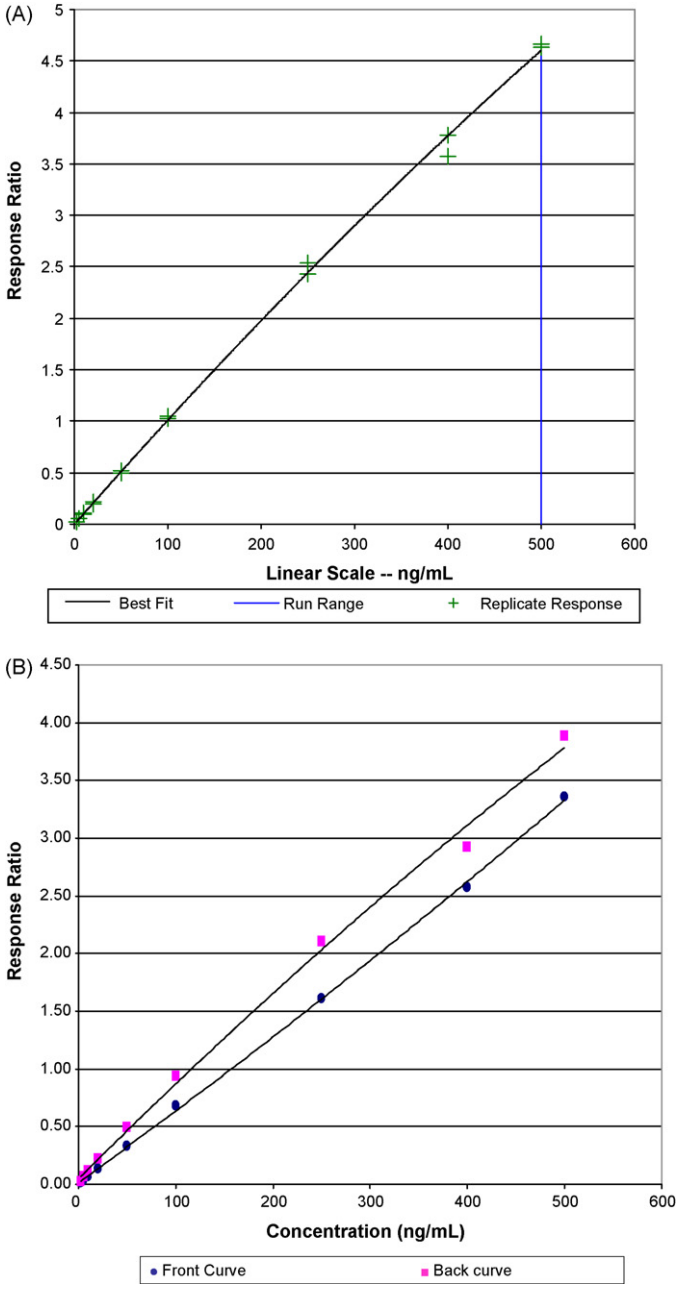


Fig. 5. (A) Calibration curves at start and end of run: interface plate conditioned prior to starting run. (B) Calibration curves at start and end of run: interface plate not conditioned prior to starting run.

volume of each sample was transferred to a 96-well polypropylene plate. A 450 μL volume of 120 ng/mL working internal standard solution, prepared in acetonitrile, was added to each sample to facilitate protein precipitation. The samples were then vortexed for 1 min and subsequently centrifuged at 2300 × *g* at room temperature for 5 min. The Tomtec was used to transfer 400 μL of the resulting supernatant to a new 96-well polypropylene plate. The 96-well plate was placed in the autosampler, set at 5 °C, and 10 μL was injected onto the column.

2.7. Regression selection

A standard curve was constructed from the peak area ratios of ixabepilone to the internal standard, BMS-212188, versus the nominal concentration of the standards. Unknown plasma sample concentrations were calculated from the equation ($y = ax^2 + bx + c$) as determined by the weighted ($1/x^2$) quadratic regression of the standard curve. A weighting factor of $1/x^2$, where x is the nominal concentration of ixabepilone at a given concentration, was applied to account for proportional increases in analyte response variance with drug concentration. The software package used to perform regression calculations is PPD Assist LIMS System, version 5.00 (PPD, Richmond, VA).

According to the most recent FDA guidelines for bioanalytical method validations, the "selection of weighting and the use of a complex regression equation should be justified" [12].

In this work, the calibration model was chosen during validation. The calibration curves from four separate batches were used to select the calibration model. While the question remains whether the calibration model should be performed in the prevalidation phase, it has been recommended that it be included during the validation using the calibration curves from several batches in order to simulate the conditions of routine analysis [13,14].

The average percent relative error (%RE) plots for both weighted and unweighted linear and quadratic regressions of the BMS-247550 inter-day calibration standards are presented in Fig. 4. The unweighted linear and quadratic regressions clearly underestimated and overestimated, respectively, the concentrations in the lower range of the curve. The linear $1/x$ regression underestimated the concentrations in the lower range of the curve while the linear $1/x^2$ regression underestimated the concentrations in the higher range of the curve. The quadratic $1/x^2$ regression provided a better balance of relative error distribution across the curve range and hence was the selected model.

The concern with using complex models, such as a quadratic regression, is the possible over-fitting of the calibration curve [14]. To evaluate the possibility of over-fitting the calibration curve, the

curve independent quality control sample data can be used. The data for the inter-day quality control samples are shown in Table 1. The results clearly show a lack of over-fitting the calibration curve.

2.8. Method validation

Validation of the method with respect to accuracy and precision was carried out according to the FDA Guidance for Industry: Bioanalytical Method Validation [12]. The accuracy and precision of the method was assessed by analyzing six QC samples at concentrations of 2.00 (LLOQ), 5.00, 30.0, 200, 400, and 4000 ng/mL. The QC sample at 4000 ng/mL was used to establish dilution integrity and was diluted 20-fold with blank matrix prior to processing. Six replicate samples at each concentration were analyzed in three separate runs. The accuracy was determined by calculating the percentage deviations (Dev%) of the predicted concentrations from their nominal values. The intra- and inter-run assay precision was determined by calculating the %CV values.

To evaluate bench-top stability, human K_2EDTA plasma quality control (QC) samples at 5.00, 400, and 4000 ng/mL were kept at room temperature for either 6 h or 24 h prior to extraction. The quality control sample at 4000 ng/mL was diluted 20-fold with blank matrix just prior to extraction. At the indicated time point, the samples were extracted and analyzed by comparison to freshly prepared standard curves. The deviations of the mean predicted concentrations of the test QC samples from the nominal concentrations were used as an indicator of the room temperature stability of ixabepilone in human K_2EDTA plasma.

The freeze–thaw stability of ixabepilone in human K_2EDTA plasma was assessed over three freeze (−20 or −70 °C)/thaw (room

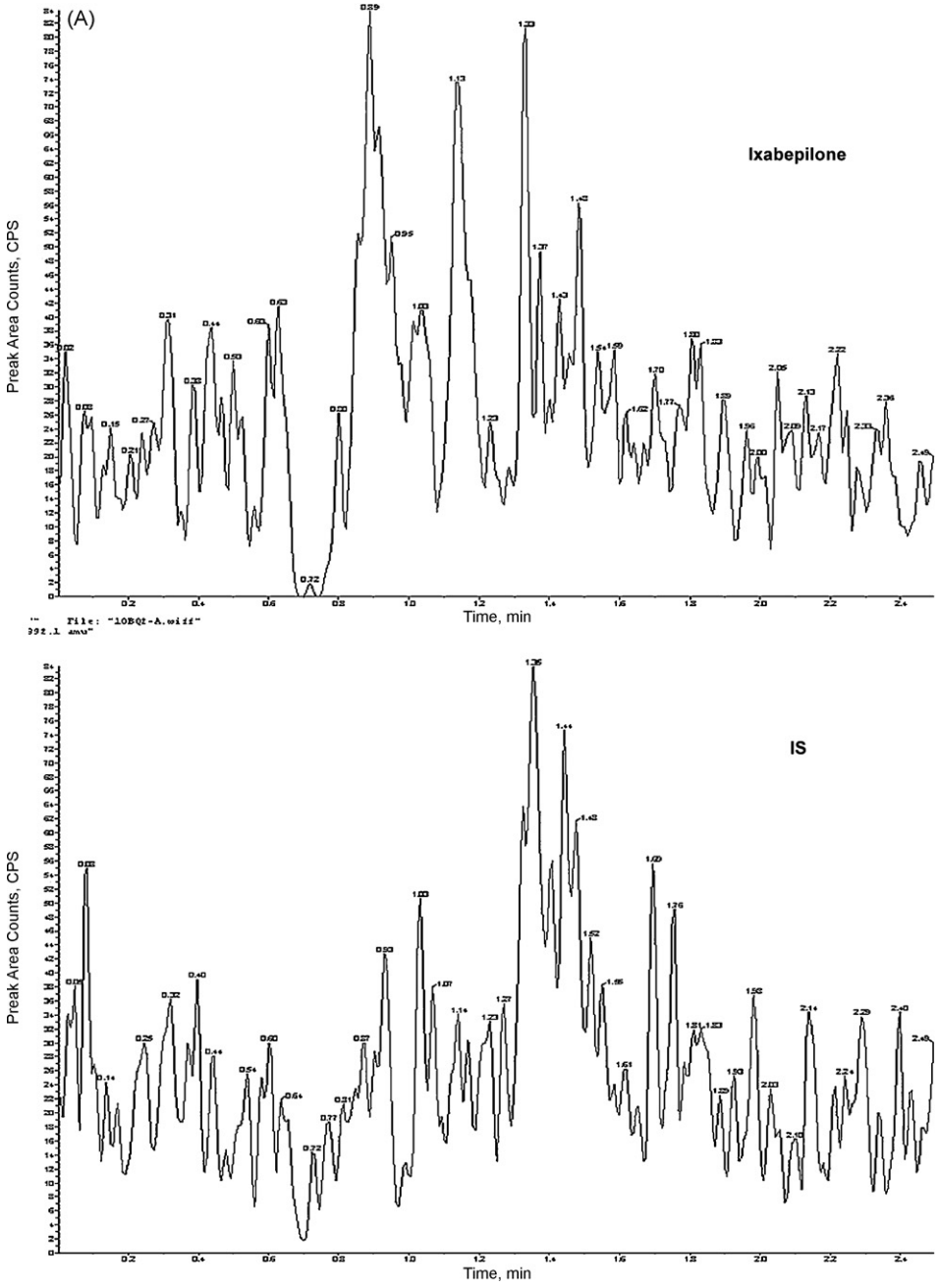


Fig. 6. (A) Chromatogram of a blank human plasma without internal standard (IS); (B) chromatogram of blank human plasma with internal standard (IS). (C) Chromatogram of a plasma sample spiked with ixabepilone at the lower limit of quantitation (LLOQ) standard (nominal = 2.00 ng/mL).

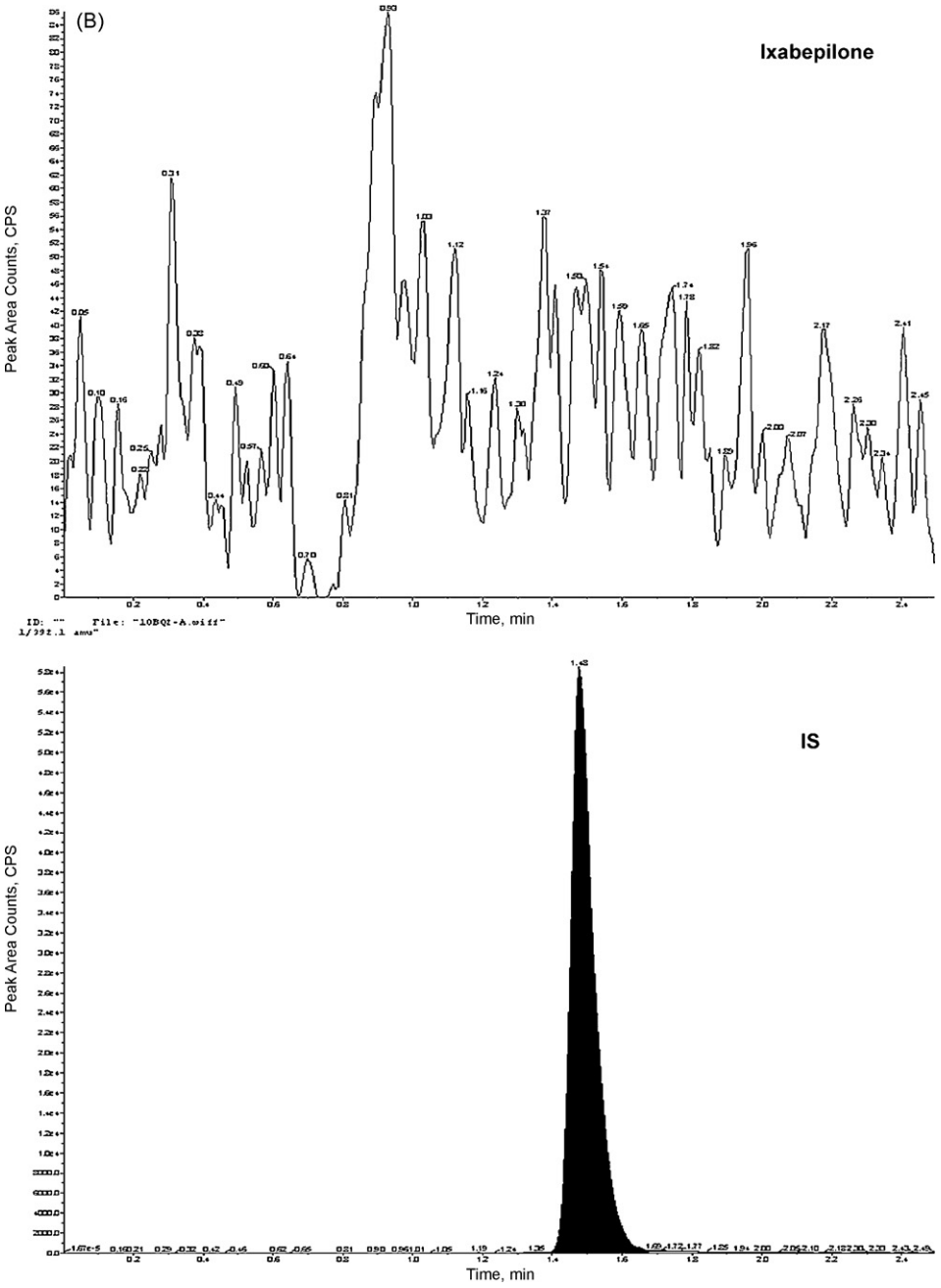


Fig. 6. (*Continued*)

temperature) cycles using QC samples at 5.00, 400 and 4000 ng/mL, in a manner consistent with typical sample analysis. After the completion of three cycles, the QC sample at 4000 ng/mL was diluted 20-fold with blank human plasma and extracted along with the other freeze/thaw QC samples.

The stability of the processed samples was assessed for 86 h at 2 to 8 °C using QC samples at 5.00, 30.0, 200 and 400 ng/mL. The above levels of QCs were extracted and analyzed along with a freshly prepared duplicate standard curve at time 0. The extracted QC samples were stored at 2–8 °C for 86 h prior to reinjection. The reinjected QC samples were quantitated against the time 0 duplicate standard curves to obtain the measured ixabepilone concentrations.

Reinjection integrity was assessed for 82 h at 2–8 °C after original sample analysis using QC samples at 5.00, 30.0, 200 and 400 ng/mL. Following extraction, the processed QCs were analyzed against a duplicate standard curve. After the successful completion of the initial analysis, the processed standard curve samples and QC samples were stored for 82 h at 2–8 °C prior to reinjection after the initial analysis completed. The reinjected QC samples were quantitated against the reinjected standard curves.

The extraction efficiency and matrix effect for ixabepilone in human plasma, expressed as a percentage, was determined at 5.00 and 400 ng/mL by comparing three sets of samples: (A) drug spiked to plasma and prepared normally (pre-extraction); (B) drug spiked after extraction of blank plasma (post-extraction), and (C) drug spiked directly into mobile phase (70:30 acetonitrile/10 mM ammonium acetate, pH 5 (v/v)) with no prior extraction. Matrix effect was calculated as the percentage decline in response between (B) and (C). Extraction efficiency was calculated as the response ratio of A/B. The recovery of the IS was determined similarly (mean peak area ratio (internal standard/analyte)) at the level of use.

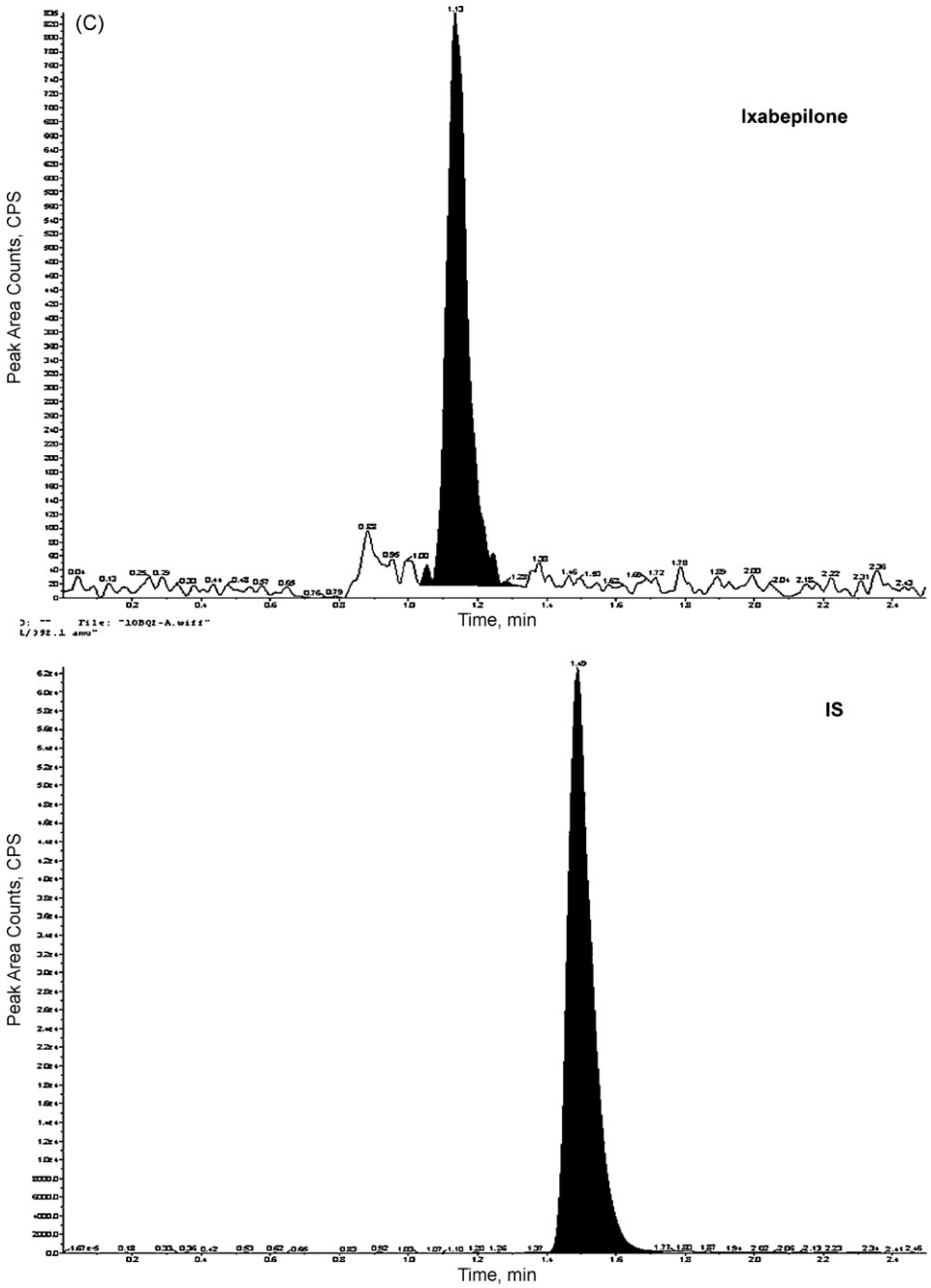


Fig. 6. (*Continued*).

3. Results and discussion

3.1. Challenges in assay development

The method presented evolved from previous versions that were validated at Bristol-Myers Squibb (BMS) and at PPD that spanned mouse, rat, rabbit, dog and human plasma matrices. Limitations of the early methods included relatively higher than acceptable run failure rates during sample analysis, regardless of species. During sample analysis, duplicate calibration curves are analyzed such that one curve was analyzed at the beginning and the other at the end of the run, thus bracketing the samples. The failed runs were largely the result of curve splitting where the responses of the first curve did not match that of the second curve. Additionally, it was observed that the responses of the first and second curves became more similar in run-batches that contained a large number of samples or if multiple batches were injected back to back. These observations, along with the use of an analogue internal standard, lead to the thinking that matrix effects were responsible for the variable responses. In brief, modifications to previous methods included: using different extraction techniques, such as liquid–liquid and solid-phase extractions, testing a variety of HPLC columns, addition of guard columns, column switching, gradient elution and isocratic elution. However, none of the modifications fully corrected the variable response problem. Additionally, a human urine assay was developed and found to experience the same trend in poor reproducibility as seen in the plasma assay. The fact that all efforts to reduce plasma matrix effects did not correct the problem and that urine assay also showed same trend suggested that matrix effects were not the problem. Instead, adsorption of

Table 2
Accuracy and precision for ixabepilone quality control (QC) samples in human K_2EDTA plasma.

Run no.	LLOQ QC (2.00 ng/mL)	Low QC (5.00 ng/mL)	GM[a] QC (30.0 ng/mL)	Mid QC (200 ng/mL)	High QC (400 ng/mL)	Dilution QC (×20) (4000 ng/mL)
1	1.94	5.58	29.19	219.86	405.34	3537.64
	2.15	5.51	31.06	203.28	421.13	4102.15
	2.27	4.86	~34.59	213.27	404.33	3923.10
	1.89	4.68	28.18	200.78	400.28	3972.53
	2.05	4.72	33.61	184.42	408.29	3939.64
	2.07	4.65	32.96	186.54	389.20	3571.98
2	1.87	4.54	26.73	171.08	340.27	3425.69
	1.71	4.74	27.43	184.19	351.53	3365.88
	1.95	4.69	27.48	177.52	352.42	3524.34
	1.84	4.58	26.76	174.99	369.38	3637.24
	1.91	4.67	25.90	175.27	355.50	3456.48
	2.09	4.71	26.04	175.62	373.58	~3385.59
3	~1.64	~4.19	28.52	183.35	355.57	3531.88
	~1.69	4.90	28.68	186.17	363.99	3693.57
	1.65	5.11	28.61	194.13	364.82	3700.82
	1.85	4.57	28.74	188.95	378.87	3725.42
	1.72	4.94	29.52	186.21	371.66	3757.11
	1.82	4.46	28.41	195.66	358.14	3619.71
4	N/A	4.84	27.43	195.18	392.17	3886.22
	N/A	5.48	31.95	214.15	418.67	4223.86
	N/A	4.92	31.11	211.29	406.33	4133.76
Mean	1.90	4.82	29.19	191.52	380.07	3719.74
SD	0.18	0.35	2.47	14.32	24.59	254.31
%CV[b]	9.40	7.25	8.48	7.48	6.47	6.84
%Nominal	94.78	96.49	97.28	95.76	95.02	92.99
%Deviation	−5.22	−3.51	−2.72	−4.24	−4.98	−7.01

(~) Values are outside ±15% of nominal values.
[a] GM: geometrical mean.
[b] Data summary are based on inter-run data from four accuracy/precision runs.

Table 3
Stability data of ixabepilone QC samples in human K_2EDTA plasma.

	Nominal Conc. (ng/mL)	Mean found conc. (ng/mL)[a]	Precision, RSD%	Accuracy (%)[b]
Bench-top stability, 6 h, $n = 6$	5.00	4.46	3.37	−10.8
	400	367	2.11	−8.28
	4000	3550	3.32	−11.2
Freeze/thaw stability at −70 °C, 3 cycles, $n = 6$	5.00	4.26	5.13	−14.8
	400	376	2.18	−6.09
	4000	3550	1.87	−11.2
Freeze/thaw stability at −20 °C, 3 cycles, $n = 6$	5.00	4.17	2.82	−16.6
	400	367	2.17	−8.26
	4000	3680	1.59	−7.97
Long-term stability at −70 °C, 914 days, $n = 3$	5.00	5.04	0.822	0.80
	400	402	0.535	0.50
	2000[c]	2040	2.20	−9.67
Long-term stability at −20 °C, 57 days, $n = 3$	5.00	4.72	6.27	−5.54
	400	371	2.19	−7.24
	4000	3650	7.50	−8.87

[a] Calculated from the weighted least-square regression curve, mean values are reported here.
[b] Expressed as [(mean observed concentration-nominal concentration)/nominal concentration)] × 100.
[c] Long-term stability at −70 °C was established for 1127 days at this concentration level.

Table 4
Processed sample stability and reinjection integrity, ixabepilone in human K_2EDTA plasma.

Sample condition	Nominal Conc. (ng/mL)							
	5.00		30.0		200		400	
	Pred. Conc.[a]	% Dev[b]	Pred. Conc.[a]	% Dev[b]	Pred. Conc.[a]	% Dev[b]	Pred. Conc.[a]	% Dev[b]
Processed sample stability, n = 6								
0 h[c]	5.00	−0.0326	31.6	5.33	201	0.681	405	1.19
86 h @ 2–8 °C	5.59	11.9	32.1	7.06	217	8.45	441	10.3
Reinjection integrity, $n = 6$								
0 h[c]	5.00	−0.0326	31.6	5.33	201	0.681	405	1.19
82 h @ 2–8 °C	5.04	0.757	29.2	−2.61	196	−1.77	398	−0.420

[a] Pred. Conc.: predicted concentration. The mean results are reported here. All concentrations were rounded to three significant figures.
[b] %Dev was calculated by Watson LIMS based on the measured concentration values against nominal values.
[c] Mean values from original injection.

Table 5
Comparison of the results from BMS and PPD for QC prepared at PPD and BMS and incurred samples.

Sample ID	Mean results (M1) from BMS (ng/mL)	Mean results (M2) from PPD (ng/mL)	Grand mean from results of two labs (ng/mL) (Mg)[a]	(%Dev)[b]
QCs prepared at BMS				
BMS QC1	5.20	6.42	5.81	±10.5
BMS QC2	35.72	37.67	36.695	±2.6
BMS QC3	206.08	240.7	223.39	±7.7
BMS QC4	390.09	408.0	399.045	±2.2
BMS Dil QC	1983.59	2270	2126.795	±6.7
QCs prepared at PPD				
PPD QC1	6.19	4.95	5.57	±11.1
PPD QC2	37.47	35.95	36.71	±2.1
PPD QC3	240.19	198.38	219.285	±9.5
PPD QC4	419.46	398.44	408.95	±2.6
PPD Dil QC	2258.65	1933.62	2096.135	±7.8
Incurred sample				
Pool A	40.67	31.36	36.015	±12.9
Pool B	202.16	163.20	182.68	±10.7
Pool C	327.27	318.00	322.635	±1.44
Pool D	685.35	693.00	689.175	±0.55

[a] Grand mean (Mg) = (M1 + M2)/2.
[b] %Dev = [(M1 − Mg)/Mg] × 100 or [(M2 − Mg)/Mg] × 100, where M1 − Mg is equal to M2 − Mg and represents the absolute difference between the grand mean (Mg) and the individual laboratory means (M1 and M2).

ixabepilone to surfaces was likely to be the cause of variable response. One possible explanation may involve the unique closed ring structure of ixabepilone in that it could potentially interact with metal ions in the LC–MS/MS system. This interaction process would eventually reach a steady-state once all possible active sites are occupied. To verify this hypothesis, high level QC samples (400 ng/mL) were extracted and injected 20 times, using an injection volume of 35 μL (3.5 times the normal injection volume for sample analysis) immediately prior to starting a sample run in order to generate a steady-state environment. This process sig-

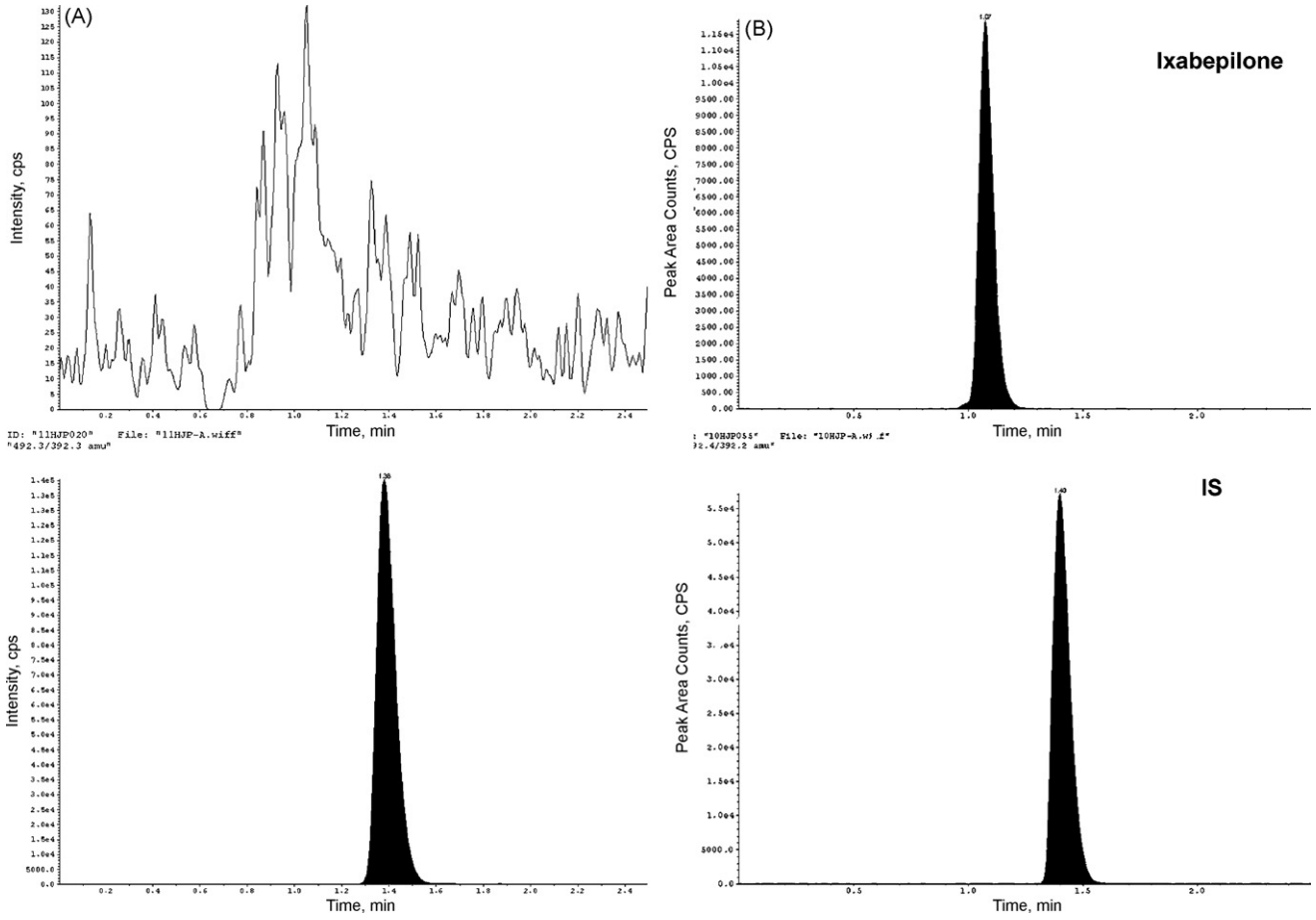


Fig. 7. (A) A typical chromatogram of a patient sample after IV dosing ixabepilone at day 1 predose. (B) A typical chromatogram of a patient sample after IV dosing with ixabepilone (16.7 ng/mL).

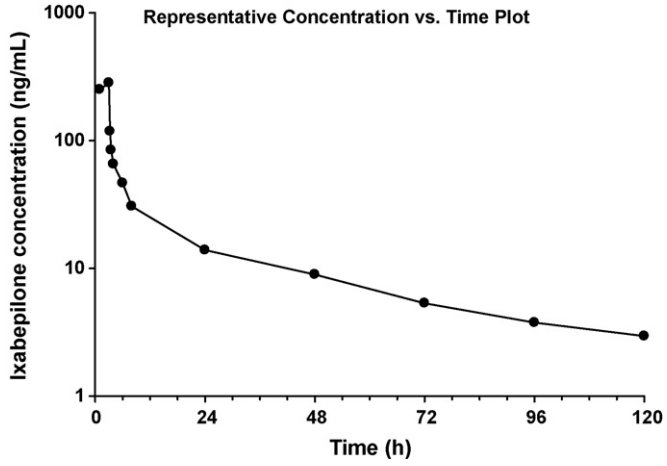


Fig. 8. Observed ixabepilone concentration time profile in plasma of a patient that received 40 mg/m^2 ixabepilone as a 3 h infusion.

nificantly reduced the variability in responses, leading to a much higher run success rate.

Although this procedure led to a workable solution, it had the disadvantage of being time consuming. An alternative approach was pursued to ascertain whether the HPLC system or the MS system or a combination of the two was responsible for the proposed analyte–LC–MS interaction. It involved infusing a neat solution (conditioning solution) containing 1.00 μg/mL of ixabepilone and 1.00 μg/mL BMS-212188 (internal standard) at 0.05 mL/min through a tee-liquid juncture, placed post-column, with a mobile phase flow rate of 0.30 mL/min for at least 10 min immediately prior to starting the analytical run. The purpose of infusing the neat solution was to reduce potential active sites on the interface plate, thus conditioning or saturating the interface plate. To test the theory, the interface plate was removed and cleaned with methanol and water and the interface plate was conditioned, by the procedure described above, immediately prior to starting the run. As can be seen in Fig. 5A, the duplicate curves are superimposable. As a control, the interface plate was removed and cleaned with methanol and water as above, and the run injected without conditioning the interface plate. As can be seen in Fig. 5B, the duplicate curves were split due to differences in responses at the start and the end of run. The conditioning appears to be related to active sites on the interface plate and not to the HPLC column as changing column, conditioning the column or cleaning the column could not correct the curve splitting issues. Therefore, the infusion of the conditioning solution immediately prior to starting a run provided a practical and convenient solution to the variability problem.

3.2. Assay validation summary

The method was fully validated to ensure the quality of clinical data. The assay specificity was established by the separation of the chemical degradants from ixabepilone. The amount of degradants through IV formulation is small, and they are not interfering ixabepilone at the retention time. Chemical degradants will be discussed in detail in the oral formulation section. The assay selectivity was established by using six different lots of human control plasma spiked with and without internal standard in order to determine whether any endogenous K_2EDTA plasma constituents interfered with the analyte or the internal standard. The degree of interference was assessed by inspection of MRM chromatograms. As shown in Fig. 6A and B, no significant interfering peaks from the K_2EDTA plasma were found at the retention time and in the ion channel of either the analyte or the internal standard. The representative chromatogram at LLOQ level is shown in Fig. 6C.

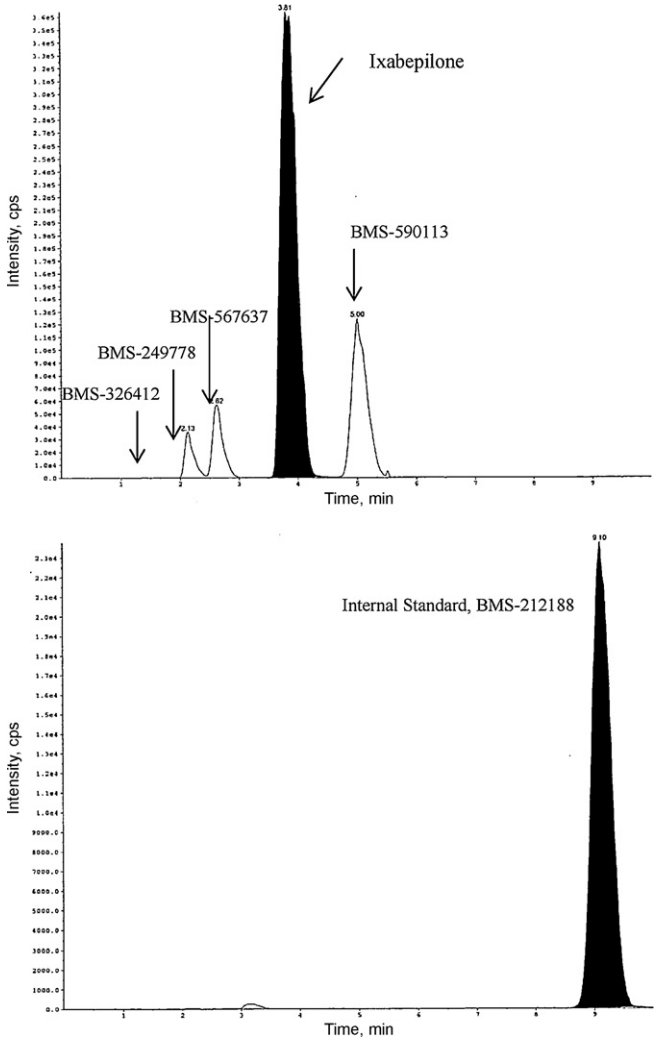


Fig. 9. Chromatographic separation of the four degradants, ixabepilone, and internal standard using a 40:60 acetonitrile/10 mM ammonium acetate pH 5.0 mobile phase and a 2.00 mm × 100 mm YMC ODS-AQ, 3 μm column at a flow rate of 0.30 mL/min.

Table 1 presents the summary of the individual standard curve data obtained in the four runs on 4 days used to validate the method. The precision and accuracy of the standard curves, at each concentration, were within ±15.0% including the lower limit of quantitation (LLOQ) whose acceptance limit is ±20.0%.

Table 2 showed intra- and inter-day precision and accuracy of QC samples for the determination of ixabepilone in human plasma. The intra-day precision was within 8.6% CV and inter-day precision was within 9.4% CV.

The freeze–thaw stability of ixabepilone in human K_2EDTA plasma was assessed over three freeze (−20 or −70 °C)/thaw (room temperature) cycles using QC samples. As shown in Table 3, the measured ixabepilone concentrations for the three cycle freeze (−70 °C)/thaw room temperature samples were within ±15% of the expected concentrations. The measured ixabepilone concentrations for the three cycle freeze (−20 °C)/thaw room temperature samples were not within ±15% of the expected concentrations for the 5.00 ng/mL QC sample. Therefore, ixabepilone was determined to be stable for at least three freeze–thaw cycles at −70 °C only.

The long-term storage stability of ixabepilone in human plasma was evaluated for up to 914 days at −70 °C using quality control. The stability QC samples were analyzed against a freshly prepared stan-

dard curve. The deviations of the mean predicted concentrations of the stability QC samples from the nominal concentrations were used as an indicator of the stability of ixabepilone in human K_2EDTA plasma. In addition, 57 days of stability has been demonstrated at −20 °C, as shown in Table 3.

The stability of the processed samples was assessed for 86 h at 2–8 °C using QC samples at different levels. The reinjected QC samples were quantified against the time 0 duplicate standard curves to obtain the measured ixabepilone concentrations. As shown in Table 4, the processed sample concentrations were within ±15% of the expected concentrations. The results demonstrate that ixabepilone was stable in processed samples for at least 86 h when stored at 2–8 °C.

The reinjection integrity was assessed for 82 h at 2–8 °C after original sample analysis using QC samples. The reinjected QC samples were quantitated against the reinjected standard curves. The deviations of the predicted concentrations of the reinjected QC samples from their nominal values ranged from −2.61 to 0.757%. As shown in Table 4, the reinjected QCs demonstrate the reinjection integrity of processed samples for at least 82 h when stored at 2–8 °C.

The extraction efficiency and matrix effect for ixabepilone in human plasma, expressed as a percentage, was determined at 5.00 and 400 ng/mL. The recovery of ixabepilone was 94.6 and 101% at the levels tested and that of the IS was 94.6 and 95.9%. The results from the matrix effects evaluation for ixabepilone was 1.20 and 1.19 at the levels tested and that of the IS was 1.32 and 1.49.

Furthermore, a cross-validation was conducted using QC samples prepared at PPD and BMS, respectively. QC samples from both labs and incurred samples were analyzed at both labs by analysts blinded to the concentration levels using the assay at each lab. The cross-validation results are shown in Table 5. The percentage of deviation (%dev) between the BMS and PPD analysis for the QC samples were within ±11.1% and for the incurred samples were within ±12.9% respectively, which met the cross-validation acceptance criteria. The results demonstrated that there was no significant difference between the data generated in PPD and BMS.

3.3. Application to the PK of ixabepilone following IV administration

The validated method was applied to study the pharmacokinetics of ixabepilone in later phase and life-cycle management clinical studies employing IV formulation. A typical chromatogram of a patient sample at day 1 predose is presented in Fig. 7A. And another typical chromatogram of the sample patient sample is presented in Fig. 7B with a calculated concentration of 16.7 ng/mL. Fig. 8 shows the observed ixabepilone concentration time profile in plasma of a patient that received 40 mg/m^2 of ixabepilone as a continuous 3 h infusion.

3.4. Application to the PK of ixabepilone following oral administration

The method presented here was initially developed and validated to support clinical development of an IV formulation. However, for the sake of patient convenience, oral formulations of ixabepilone are under development. Ixabepilone has been shown to be labile under highly acidic conditions; therefore, oral formulations are susceptible to chemical degradation. To overcome the acid labile issue, an enteric coated oral formulation was developed to support such human clinical studies, but it is anticipated that some degradation may still occur during the release. The structures of the degradants are shown in Fig. 2. Among them, degradants 1, 2 and 3 have the same molecular weight and the same predominant

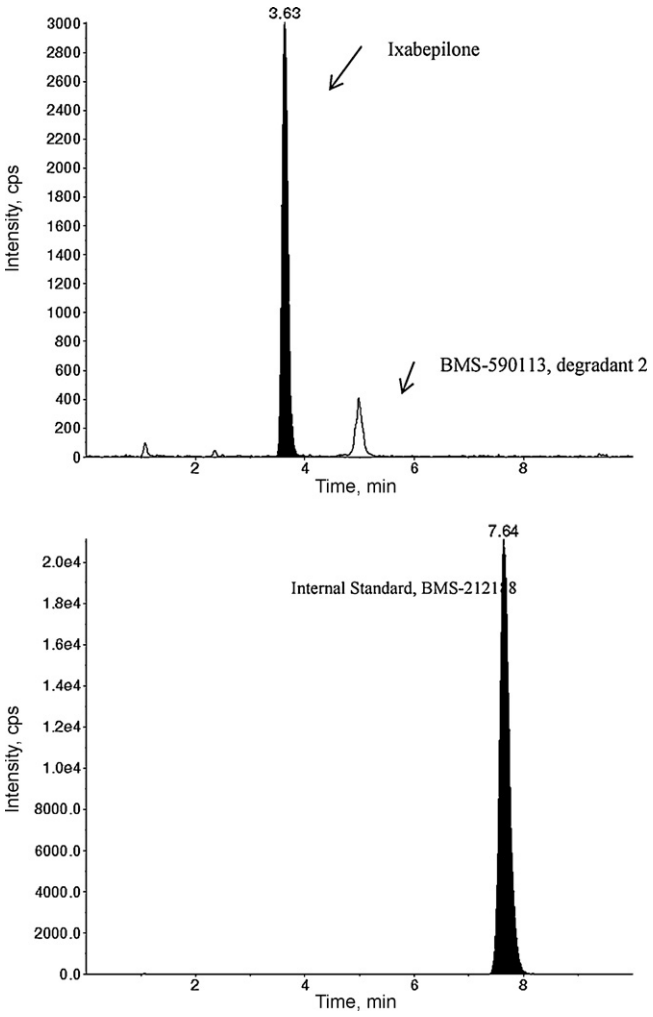


Fig. 10. A typical chromatogram of a patient sample dosed with the oral formulation of ixabepilone.

precursor → product ion transitions as ixabepilone (505 → 405). Degradant 4, a diol, has a unique molecular weight of 524.73 but potentially could undergo in-source conversion back to ixabepilone though dehydration occurring in the source. Therefore, it was imperative that the chromatography be evaluated and optimized to ensure ixabepilone was separated from the four degradants, since a unique ixabepilone MRM transition could not be identified. A neat solution was prepared containing the four degradants, ixabepilone, and the internal standard and analyzed under the method conditions presented in this paper. Under the above condition, baseline resolution was not obtained. The mobile phase conditions were modified from 70:30 acetonitrile/10 mM ammonium acetate, pH 5.0 to 40:60 acetonitrile/10 mM ammonium acetate, pH 5.0 and the run time extended to 10 min. In these conditions (Fig. 9), baseline resolution was obtained. No in-source conversion of degradant 4 to ixabepilone was observed. A unique MRM transition was established for degradant 4 and its retention time was determined to be 1.6 min. The elution times were 1.6, 2.2, 2.6, 3.5, 4.5, and 7.5 min for degradant 4, degradant 1, degradant 3, ixabepilone, degradant 2 and the internal standard, respectively. Subsequently, these method conditions were validated (data not shown) to support clinical development of the enteric coating oral formulation studies. Fig. 10 is a representative chromatogram of a patient sample dosed with the oral formulation, degradant 2 (BMS-590113) was detected in plasma.

4. Conclusions

A simple and robust LC–MS/MS method for the determination of ixabepilone in human K_2EDTA plasma was developed and validated for studies involving IV dosing. The procedure is straightforward and involves a one step protein precipitation extraction combined with LC–MS/MS detection has been developed and validated for the quantitative determination of ixabepilone in human K_2EDTA plasma, following an IV administration. Furthermore, a unique source of variance has been hypothesized that may involve in-source metal ion interaction and has been overcome by pretreatment of the source with ixabepilone and internal standard. This method is specific, accurate and precise, and samples can be prepared rapidly. Using a modified chromatography, the method may also be utilized to measure ixabepilone after oral administration.

Acknowledgements

The authors wish to acknowledge Dr. Vikram Roongta and Dr. Anne-Françoise Aubry for their thorough reviews and, Steven Dennell and John Lute who conducted earlier work on this compound. This manuscript is particularly dedicated to Glen Duncan, now deceased, who provided the original scientific leadership in developing this method.

References

[1] F.Y. Lee, R. Smykla, K. Johnston, K. Menard, K. McGlinchey, R.W. Peterson, A. Wiebesiek, G. Vite, C.R. Fairchild, R. Kramer, Cancer Chemother. Pharmacol. 63 (2009) 201.
[2] J.A. Low, S.B. Wedam, J.J. Lee, J. Clin. Oncol. 23 (2005) 2726.
[3] J. Hunt, Mol. Cancer Ther. 8 (2009) 275.
[4] F.Y. Lee, R. Borzilleri, C.R. Fairchild, A. Kamath, R. Smykla, R. Kramer, G. Vite, Cancer Chemother. Pharmacol. 63 (2008) 157.
[5] D. Schiff, P.Y. Wen, M.J. van den Bent, Nat. Rev. Clin. Oncol. 6 (2009) 596.
[6] M. Jemal, Y.-Q. Xia, Curr. Drug Metab. 7 (2006) 491.
[7] C. Aghajanian, H.A. Burris III, S. Jones, D.R. Spriggs, M.B. Cohen, R. Peck, P. Sabbatini, O.A. O'Connor, J. Clin. Oncol. 25 (2007) 1082.
[8] S.M. Gadgeel, A. Wozniak, R.R. Boinpally, Clin. Cancer Res. 11 (2005) 6233.
[9] J.H. Beumer, R.C. Garner, M.B. Cohen, S. Galbraith, G.F. Duncan, T. Griffin, J.H. Beijnen, J.H.M. Schellens, Invest. New Drugs 25 (2007) 327.
[10] B. Mansoori, D.A. Volmer, R.K. Boyd, Rapid Commun. Mass Spectrom. 11 (1997) 1120.
[11] S. Zhou, K. Cook, J. Am. Soc. Mass Spectrom. 11 (2000) 961.
[12] Food and Drug Administration, US Department of Health and Human Services, Guidance for Industry: Bioanalytical Method Validation, 2001.
[13] A.M. Almeida, M.M. Castel-Branco, A.C. Falcão, J. Chromatogr. B 774 (2002) 215.
[14] T. Singtoroj, J. Tarning, A. Annerberg, M. Ashton, Y. Bergqvist, N.J. White, N. Lindegard, N.P.J. Day, J. Pharm. Biomed. Anal. 41 (2006) 219.