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Measurement of paclitaxel in biological matrices: high-throughput liquid chromatographic–tandem mass spectrometric quantification of paclitaxel and metabolites in human and dog plasma

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Abstract

A GLP-validated, sensitive and specific LC–MS–MS method for the quantification of paclitaxel and its 6-α- and 3′-*p*-hydroxy metabolites is presented. A 0.400 ml plasma aliquot is spiked with a $^{13}C_6$-labeled paclitaxel internal standard and extracted with 1 ml methyl-*tert*.-butyl ether. The ether is evaporated and the residue is reconstituted in 130 μl of 30% aqueous acetonitrile (ACN) containing 0.1% trifluoroacetic acid. Isocratic HPLC analysis is performed by injecting 50 μl of the reconstituted material onto a 50×2.1 mm C_{18} column with an ACN–water–acetic acid (50:50:0.1) mobile phase at 200 μl/min flow. Detection is by positive ion electrospray followed by multiple reaction monitoring of the following transitions: paclitaxel (854>509 u), 6-α-hydroxy paclitaxel (870>525 u), 3′-*p*-hydroxy paclitaxel (870>509 u) and internal standard (860>509 u). Quantification is by peak area ratio against the $^{13}C_6$ internal standard. The method range is 0.117–117 n*M* (0.1–100 ng/ml) for paclitaxel and both metabolites using a 0.400 ml human or dog plasma sample. Analysis time per sample is less than 5 min.

Keywords: Paclitaxel; Hydroxy paclitaxel

1. Introduction

Paclitaxel is an antitumor agent derived from the bark of the Pacific yew tree *Taxus brevifola*. Its activity is derived from its ability to increase the assembly and stability of microtubules in dividing cells, blocking the cell cycle [1]. It presently is used in therapy for breast and ovarian cancer and is being investigated for other therapeutic indications.

Paclitaxel (PT) has two known active metabolites [2], 6-α-hydroxy paclitaxel (6OHPT) and 3′-*p*-hydroxy paclitaxel (3OHPT). The structures of these compounds, along with the labeled internal standard, are shown in Fig. 1.

Pharmacokinetic analysis of PT generally is performed using high-performance liquid chromatography (HPLC) with UV detection [3–8]. These

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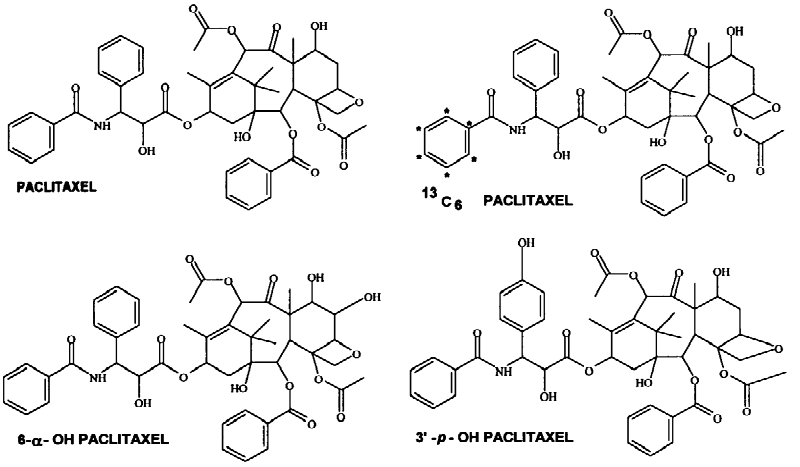

Fig. 1. Structures of paclitaxel, hydroxylated metabolites, and internal standard (* = ^{13}C substitution).

methods have reported lower limits of quantification (LLOQ) in the 5–20 n*M* range. In a related [9] paper, an LC–MS–MS method is described which is widely applicable to the rapid analysis of PT and its hydroxy metabolites in a variety of matrices with minimal sample workup and reasonable quantification limits.

The LLOQ can be misleading when reported simply in terms of sample concentration, because it does not give an indication of the sample size required or the proportion of the analyte in the sample actually injected for analysis, both critical factors when designing an assay for a particular purpose. To compare the innate sensitivity of different assays, the actual amount of sample injected at the LLOQ should be calculated. For example, two of the published PT assays referenced above [4,8] report LLOQ values of 12 n*M* (10 ng/ml), but one requires 0.100 ml plasma and injects 0.300 nmol PT on-column while the other requires 1.00 ml plasma and injects 6 nmol on-column, 20-times as much. This is not to imply that one method is superior to the other, but merely that the two methods were optimized for different uses with the tools available.

With these points in mind, low mass-on-column assays have an inherently greater flexibility. Less sample is required and sample work up often is quicker. One can trade some low-end sensitivity for smaller sample volume or multiple injections, for example. Also, the less sample placed in the system, the cleaner the system will stay, minimizing problems such as increasing column back pressure and detector contamination.

2. Experimental

2.1. Standards

Paclitaxel ($C_{47}H_{51}NO_{14}$, 99.4% purity by HPLC), 6α-hydroxy paclitaxel ($C_{47}H_{51}NO_{15}$, purity 93% by HPLC) and 3′-*p*-hydroxy paclitaxel ($C_{47}H_{51}NO_{15}$, purity 84% by HPLC) were purchased from the Gentest Corporation (Woburn, MA, USA). NaPro BioTherapeutics (Boulder, CO, USA) provided $^{13}C_6$-paclitaxel ($C_{47}H_{51}NO_{14}$). The purity of this standard is 99.5% as determined by HPLC and 99.9% $^{13}C_6$-labeled as determined by mass spectrometry (MS). All other solvents and chemicals were reagent grade or better.

Standard calibrator (SC) samples were prepared by spiking blank human and dog plasma with methanolic solutions of PT, 6OHPT, and 3OHPT followed by serial dilutions using blank plasma. The SC concentrations were 0, 0.117, 0.351, 0.878, 1.17, 3.51, 8.78, 11.7, 35.1, 87.8, and 117 n*M* (0, 0.1, 0.3, 0.75, 1, 3, 7.5, 10, 30, 75, and 100 ng/ml). Quality control (QC) samples were prepared similarly using

a separate weighing of analytes to give concentrations of 0.117, 0.351, 35.1, and 117 n*M* (0.1, 0.3, 30, and 100 ng/ml). Final concentrations of the metabolites were corrected to reflect the purity of the starting material.

2.2. Sample preparation (human and dog)

A 400-μl aliquot of each plasma sample was transferred to a 2-ml polyethylene microcentrifuge tube. A 50-μl aliquot of internal standard solution ($^{13}C_6$-PT in water, 46 n*M*) was added to each tube and the contents briefly mixed by vortexing. A 1.0-ml aliquot of methyl-*tert*.-butyl ether (MTBE) was added to each tube. Samples were vortexed for 10 min, followed by centrifugation (≈3 min at >10 000 rpm) to separate the phases. Samples then were placed in a freezer at ≤−60 °C for approximately 45 min, or until the plasma layer was completely frozen. The MTBE layer was decanted into a second microcentrifuge tube and taken to dryness under nitrogen in a water bath at 30 °C (about 10 min). Samples were reconstituted with 0.13 ml of reconstitution solution [30% acetonitrile (ACN) in water containing 0.1% trifluoroacetic acid] and transferred to 0.2-ml micro-inserts in autosampler vials. A 50-μl aliquot of each sample was injected onto the HPLC column.

2.3. HPLC

The HPLC system comprised an LC-10ADvp pump and SCL-10Avp controller (Shimadzu, Columbia, MD, USA) fitted with an Upchurch micromixer (p/n U-466, Upchurch Scientific, Oak Harbor, WA, USA), a CH-150 column oven (Eldex Labs., Napa, CA, USA), and an ISS-200 autosampler (Perkin-Elmer, Norwalk, CT, USA). Isocratic chromatography was performed using a Zorbax 50×2.1 mm SB-C_{18} column with 5 μm packing (p/n 860975-902, Agilent Technologies, Palo Alto, CA, USA). The mobile phase was 0.1% acetic acid in HPLC-grade ACN–water (1:1, v/v). The flow-rate was 0.200 ml/min and the column temperature was 50 °C. Typical retention times for PT, 6OHPT, 3OHPT, and internal standard were 2.9, 2.0, 1.7, and 2.9 min, respectively.

2.4. Mass spectroscopy

The interface was a Perkin-Elmer TurboIonspray operated at 400 °C. The mass spectrometer was an API-365 (PE-Sciex, Toronto, Canada) operated in the positive ion, multiple reaction monitoring (MRM) mode, observing the transitions for PT, 6OHPT, 3OHPT, and internal standard (854>509, 870>525, 870>509, and 860>509 u, respectively). Dwell time for each transition was 200 ms. Mass spectrometer response was optimized for the PT transition, with the same tune parameters used for all three analytes and the internal standard. Both the first and third quadrupoles were operated under lowered resolution conditions to further increase sensitivity.

2.5. Description of the validation

The validation was performed under US Food and Drug Administration (FDA) guidelines for Good Laboratory Practice (GLP) [10,11] with approved study and method protocols in place before the validation was started. Validation runs were conducted on three separate days. Each validation run comprised a set of 10 SCs bracketing the concentration range, QC samples at four concentrations (n=6 at each concentration), blank, stability, and freeze–thaw samples. SCs were analyzed at the beginning of each validation run. Other samples were distributed randomly throughout the run. In order to bring the total run size up to that encountered in an actual large analytical run, pooled plasma samples from previously analyzed dog subjects were extracted and analyzed throughout the run, bringing the total number of samples analyzed to about 250 on each day. Dog and human samples were analyzed in a single analytical run, using independently prepared sets of SCs and QCs for each species.

Concentrations of the three analytes in QC, recovery, stability, and freeze–thaw samples were determined by back-calculation of the observed peak area ratios of the analyte and internal standard from the best-fit linear regression line of the SC samples, using inverse concentration squared ($1/x^2$) weighting.

3. Results and discussion

3.1. Method development

Development of any assay involves a series of compromises and the balancing of a large number of dependent variables. Before setting the final form, it is helpful to first decide the assay performance requirements and scope of application. Major goals in the development of this assay were the ability to rapidly process large numbers of clinical or toxicological plasma samples under GLP guidelines at low PT/metabolite levels from a relatively small (e.g., $\leq$1 ml) sample volume.

Preliminary method development for both HPLC and MS was performed in parallel to shorten development time. Use of short HPLC columns and gradient elution established likely stationary and mobile phase candidates. An acidic mobile phase was required to suppress formation of the $M+Na^+$ molecular ion in favor of the $M+H^+$ ion; acetic acid was chosen instead of trifluoroacetic acid because the latter caused a significant suppression of absolute signal. Optimum MS conditions for the target analyte response were established using continuous flow and direct loop injection while running the chosen mobile phase components, then estimating the signal/noise quantification limit of the assay in terms of fmol on-column. Knowing this, an appropriate combination of plasma concentration, sample volume, column dimensions, and retention times could be chosen for a workable assay.

The LLOQ of 0.117 n*M* in this method corresponds to about 18 fmol PT on-column, using the 50×2.1 mm I.D. column, providing a signal/noise ratio of about 20:1. The narrow bore column provides a significant ($\approx$5×) increase in sensitivity compared to a traditional 4.6 mm column, and the reduced flow-rate obviates the need for post-column splitting of the HPLC effluent.

HPLC separation conditions were optimized to provide the shortest analysis time consistent with good accuracy and reproducibility. Suppression of analyte ionization by background substances in the sample extract was examined by injecting a blank plasma extract on the HPLC column while adding a constant, low concentration of PT to the mobile phase between the column and the MS interface. It took about 1.5 min for the PT signal to return to a level baseline. To avoid ion suppression at the solvent front, the retention time for the least retained component was then adjusted to be slightly longer than 1.5 min.

The final method conditions were chosen for several reasons. First, they allow the use of relatively small amounts of sample, which can be an important consideration when limited sample volumes are available. Second, they permit reinjection of the extracted sample if questions arise concerning chromatography or MS response in the course of an analytical run. Third, they allow a linear dynamic range of 1000. Our observations indicate that one of the causes of nonlinearity at the high end of PT assays is formation of PT dimers and trimers in the interface at high concentrations. Keeping low absolute concentrations in the interface minimizes this effect and gives extremely linear response over the entire assay range. Method performance is aided significantly by the use of the stable isotopically labeled internal standard.

The performance of the narrow-bore column was enhanced and protected by using liquid–liquid extraction as the sample preparation technique. Not only does liquid–liquid extraction produce a spectroscopically clean sample, but few nonvolatile materials are introduced onto the column and MS system. Extremely clean samples are essential for long, unattended HPLC runs on narrow-bore columns, where blockage and subsequent increases in back pressure can compromise the assay. Also, when hundreds or thousands of samples are injected, clean samples minimize signal degradation due to fouling of the active elements in the MS system. High throughput demands minimal downtime for source cleaning and subsequent recalibration and sensitivity checks. With the current assay, about 2000 samples can be injected before source cleaning becomes necessary.

PT adheres strongly to most surfaces; carryover and ghost peaks were major problems during the early phase of method development. Problems were traced to the autosampler, and were eliminated by using pure isopropanol as a needle wash solvent.

MS sensitivity to PT and its metabolites depends on a relatively high interface temperature. For optimum response it was found that the TurboIonspray

interface had to be set at 400 °C for a 200 μl/min mobile phase flow-rate. Lower temperatures resulted in loss of most of the signal as well as a significant increase in peak tailing. To further increase sensitivity, both the first and third quadrupoles were operated at less than unit resolution. Although this usually is not done, the combination of the relatively high molecular mass of PT/metabolites, MRM selectivity, and the cleanliness of the extraction resulted in clean backgrounds with no significant interferences.

3.2. Accuracy and precision

Accuracy and precision were assessed for both human and dog plasma samples. The predetermined criteria for acceptance were that the SCs had to be accurate within ±15% of their nominal values as determined by the best-fit regression line except at the LLOQ, where ±20% was acceptable. The correlation coefficient (r^2) also had to be 0.95 or better. For the QCs, both accuracy (recovery, %) and precision (RSD, %) of the back-calculated concentrations had to be within ±15 % of the nominal values except at the LLOQ, where ±20% was acceptable. These criteria applied to both within- and between-run results. All criteria were met for all analytes in both species on all validation days. No samples were excluded from these calculations in any of the runs.

SCs containing all three analytes in both dog and human plasma were analyzed on each validation day. A summary of the regression results for human and dog on all 3 days is shown in Table 1. Fig. 2 shows representative results (data points and best-fit line) of the three analytes in human plasma on day 1. Results were similar for both human and dog on all 3 days.

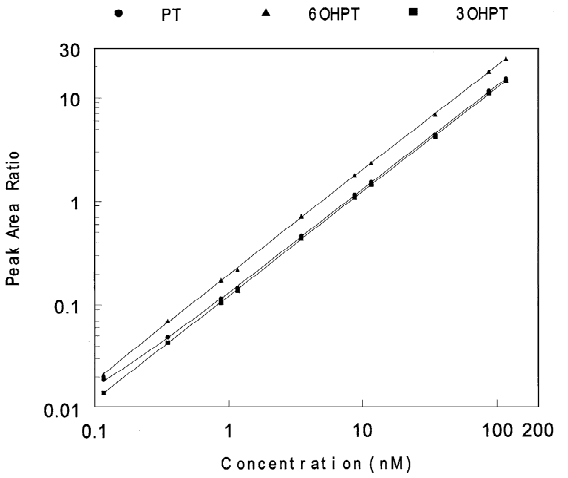

Fig. 2. Standard curves (peak area ratio of analyte and internal standard vs. analyte concentration) for paclitaxel and hydoxylated metabolites generated from standard calibrator (SC) samples in human plasma.

Response of the assay for both human and dog plasma for all three analytes was both accurate and reproducible over the course of the validation. Corre-

Table 1
Slope, intercept, and correlation coefficient values of the best-fit lines through the standard calibrators (SCs) for PT, 6OHPT and 3OHPT for human and dog plasma on validation days 1–3

	PT			6OHPT			3OHPT		
	Slope	*y*-Intercept	r^2	Slope	*y*-Intercept	r^2	Slope	*y*-Intercept	r^2
Human									
Day 1	0.1259	0.0035	0.9997	0.1953	−0.0015	0.9996	0.1200	0.0001	0.9997
Day 2	0.1335	0.0016	0.9990	0.2053	0.0001	0.9992	0.1318	0.0005	0.9990
Day 3	0.1312	0.0019	0.9991	0.1953	0.0007	0.9988	0.1229	−0.0002	0.9995
Average	0.1300	0.0024	–	0.1988	−0.0002	–	0.1247	0.0001	–
Dog									
Day 1	0.1329	0.0042	0.9994	0.1841	0.0009	0.9995	0.0982	−0.0005	0.9984
Day 2	0.1429	0.0021	0.9992	0.1941	−0.0018	0.9994	0.1053	−0.0008	0.9993
Day 3	0.1406	0.0025	0.9994	0.1853	0.0000	0.9992	0.0947	0.0011	0.9989
Average	0.1388	0.0029	–	0.1876	−0.0003	–	0.0994	−0.0001	–

SC concentrations at 0.117, 0.351, 0.878, 1.17, 3.51, 8.78, 11.7, 35.1, 87.8, and 117 n*M*.

lation coefficients for the best-fit lines were generally $r^2 \geq 0.999$, and the peak area ratio at the *y*-intercept was small (15% or less) compared to the peak area ratio of the analyte at the limit of quantification.

The accuracy and precision of the method were judged by the back-calculated concentrations obtained from the QC samples on each analysis day. The QC concentrations chosen were in accordance with FDA guidelines: one at the lower quantification limit (0.117 n*M*), one within 3× the lower quantification limit (0.351 n*M*), one midrange (35.1 n*M*) and one at the upper limit of quantification (117 n*M*). Accuracy and precision criteria were met easily for all analytes in both matrices on all days. Table 2 summarizes the within- and between-day accuracy and precision values for the three analytes in both matrices. Mean accuracy±mean RSDs for human QCs (averaging all concentrations) were 103±5.6, 100±4.6, and 99±5.1% for PT, 6OHPT, and 3OHPT, respectively. For dog QCs the results were 97±5.5, 98±5.2, and 98±6.6% for PT, 6OHPT and 3OHPT, respectively.

3.3. Detection limits

Fig. 3 shows the superimposed MRM signals of PT, 3OHPT, 6OHPT, and internal standard (off-scale) for a typical 0.117 n*M* (LLOQ) human plasma

Table 2
Accuracy and precision of quality control (QC) samples for human and dog plasma

	Concentration (n*M*)											
	PT				6OHPT				3OHPT			
	0.117	0.351	35.1	117	0.117	0.351	35.1	117	0.117	0.351	35.1	117
Human												
Day 1												
Mean (%)	96	99	106	102	110	106	102	97	104	100	101	99
RSD %	7.6	6.0	5.1	4.1	4.1	4.5	6.0	2.7	6.3	4.5	3.2	2.9
Day 2												
Mean (%)	105	105	103	98	101	97	99	93	98	97	96	93
RSD (%)	7.3	2.2	3.2	2.6	5.6	3.0	3.1	1.5	3.8	3.7	2.8	2.2
Day 3												
Mean (%)	111	105	107	97	102	100	103	95	101	104	103	95
RSD (%)	2.1	7.7	3.7	2.0	4.4	3.3	4.3	1.5	10	3.0	5.2	1.9
Between day												
Mean (%)	104	103	105	99	104	101	101	95	101	100	100	95
RSD (%)	8.4	6.0	4.2	3.7	6.0	5.1	4.7	2.6	7.3	4.6	4.8	3.5
Dog												
Day 1												
Mean (%)	87	94	99	99	95	97	98	100	105	100	95	96
RSD (%)	6.8	2.4	5.1	2.6	3.8	3.2	4.0	4.9	7.5	6.9	4.3	4.9
Day 2												
Mean (%)	102	99	96	97	107	100	94	98	108	100	90	94
RSD (%)	10	4.3	1.7	2.5	4.3	4.6	2.3	8.7	6.2	6.7	2.3	10
Day 3												
Mean (%)	97	99	98	98	101	98	96	97	98	96	94	97
RSD (%)	9.8	3.9	3.4	3.3	3.8	5.2	3.5	5.1	9.5	9.3	4.0	4.0
Between day												
Mean (%)	95	97	98	98	101	98	96	98	104	99	93	96
RSD (%)	11	4.3	3.7	2.9	6.3	4.5	3.7	6.2	8.2	7.5	4.0	6.7

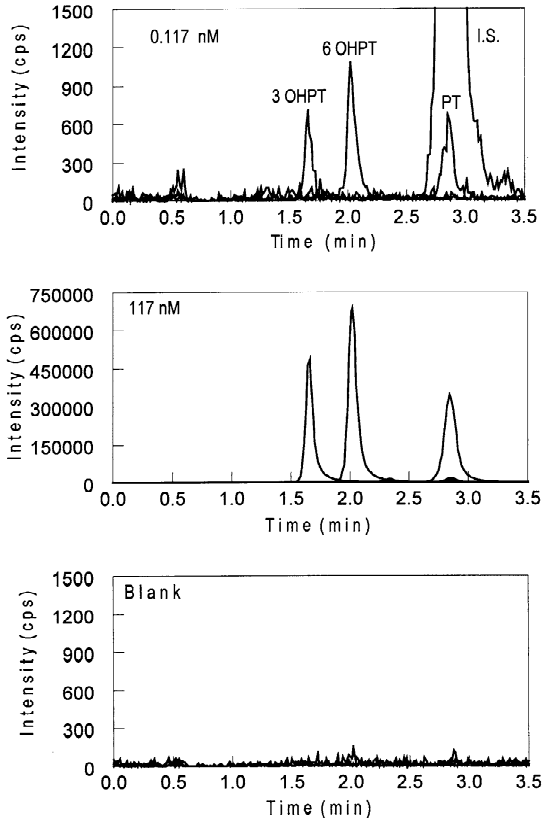

Fig. 3. Typical MRM results for paclitaxel and its hydroxylated metabolites. Top: Superimposed plots at the lower limit of quantification (LLOQ); middle: upper limit of quantification (ULOQ); bottom: blank plasma extract.

sample, a 117 n*M* sample (upper quantification limit) and a blank plasma extract. The low background and lack of interferences allowed accurate quantification at low levels.

3.4. Stability testing

Sample stability was tested for the following: plasma freeze–thaw cycles, plasma short-term room temperature exposure, and extracted sample stability.

3.4.1. Freeze–thaw

QC human and dog samples (0.117, 35.1, and 117 n*M*) were frozen (≤−60°C) and thawed three times, then frozen again until analysis was performed. Each QC was analyzed in triplicate on each validation day. Freeze–thaw results are shown in Table 3. Freeze–thaw cycling did not affect the recovery or accuracy of the assay.

3.4.2. Room temperature stability

QC human and dog samples (0.117, 35.1, and 117 n*M*) were left out on the bench (≈21°C) for 4 h, then frozen until analysis. This time period was chosen to represent the maximum time such samples would be at room temperature during a 250 sample run preparation. The between-run results are shown in Table 4. The results were all acceptable. It was observed that the values for 3OHPT for the human samples at the 35.1 and 117 n*M* concentrations averaged somewhat lower than the other stability sets. This was apparent especially on the first validation day. It suggests that 3OHPT may be somewhat less stable than the other analytes at room temperature, and that workup time should be minimized.

3.4.3. Autosampler stability

The stability of the analytes in the sample matrix in autosampler vials at room temperature was examined. Four QC samples from each run were

Table 3
Between-run freeze–thaw stability data for quality control (QC) samples for human and dog plasma

	Concentration (n*M*)								
	PT			6OHPT			3OHPT		
	0.117	35.1	117	0.117	35.1	117	0.117	35.1	117
Human									
Mean (%)	99.9	104.2	100.9	108	99.7	96.6	102.2	99.3	95.5
RSD (%)	10	1.7	2	5.1	1.4	3	10.3	2.9	2.7
Dog									
Mean (%)	100.1	99.2	97.9	99.6	95.7	96.5	98.2	93.1	93.3
RSD (%)	8.6	2.7	3.5	4.1	3	4.6	9.1	4	3.5

Table 4
Between-run room temperature stability data for quality control (QC) samples for human and dog plasma

	Concentration (n*M*)								
	PT			6OHPT			3OHPT		
	0.117	35.1	117	0.117	35.1	117	0.117	35.1	117
Human									
Mean (%)	104	104	100	103	97	91	95	89	85
RSD (%)	8.3	2.8	3.9	5.7	4.6	6.6	12.4	10.4	10.1
Dog									
Mean (%)	96	97	98	101	95	97	104	93	94
RSD (%)	10.1	3.2	2	11.6	3.2	2	9	2.8	2.7

reinjected at the end of the run to assess stability. No degradation of the analytes was observed. In addition, 10 SC and four QC samples from days 1 and 2 were stored at 5 °C and reinjected at the end of the day 3 run. Table 5 shows the accuracy values for human SCs and QCs for day 3 compared to the reinjected day 2 samples. The data indicate that extracted samples can be stored for extended periods without analyte degradation.

3.5. Effect of dilution

During normal analysis, samples are encountered occasionally that exceed the upper limit of quantification (ULOQ) for a method. Common practice is to dilute the sample with blank plasma so that it falls within the linear range of the method. Dilution effects were examined by preparing a QC sample at a concentration of 351 n*M* (3× the ULOQ). This sample was diluted 10-fold with blank plasma and analyzed. Results (Table 6) indicated that dilution has no effect on accuracy of PT or its metabolites.

3.6. Long-term assay performance

This analytical method has been used to assay more than 10 000 dog plasma samples and a smaller number of human plasma samples. Because the assay

Table 5
Autosampler stability of standard calibrator (SC) and quality control (QC) human plasma samples

Sample-n*M*	PT			6OHPT			3OHPT		
	Run 2	Run 3	Difference*	Run 1	Run 3	Difference*	Run 2	Run 3	Difference*
SC-0.117	101.4%	104.9%	3.4	100.2%	108.0%	7.5	98.4%	104.5%	6.1
SC-0.351	98.7%	96.5%	2.3	102.2%	99.7%	2.5	106.8%	106.1%	0.7
SC-0.878	97.3%	100.4%	3.2	96.0%	100.5%	4.6	98.2%	101.0%	2.8
SC-1.17	94.2%	96.2%	2.2	96.9%	101.7%	4.9	96.6%	99.5%	3
SC-3.51	97.8%	106.3%	8.3	97.1%	104.3%	7.2	98.9%	105.2%	6.2
SC-8.78	99.9%	103.1%	3.2	102.8%	103.5%	0.7	99.1%	103.2%	4
SC-11.7	103.4%	102.2%	1.2	101.9%	101.7%	0.2	101.1%	103.2%	2
SC-35.1	96.0%	102.4%	6.4	95.0%	105.8%	10.7	93.1%	101.9%	8.9
SC-87.8	103.6%	107.5%	3.7	101.2%	105.6%	4.3	103.2%	107.2%	3.8
SC-117	107.8%	106.0%	1.7	106.9%	106.3%	0.5	104.7%	100.4%	4.2
QC-0.117	101.2%	99.1%	2.1	94.7%	95.8%	1.1	97.6%	115.6%	16.9
QC-0.351	105.3%	113.6%	7.6	99.9%	107.5%	7.3	94.3%	108.7%	14.2
QC-35.1	100.0%	102.3%	2.3	99.9%	102.8%	2.8	98.3%	102.5%	4.1
QC-117	93.5%	96.1%	2.8	94.7%	96.4%	1.8	93.7%	94.8%	1.1

Run 2 data are for day 2 samples rerun on day 3; compare to fresh run 3 samples run on day 3.
* Calculated prior to rounding % assay values to three significant figures.

Table 6
Tenfold dilution of 351 n*M* spiked plasma samples

	PT	6OHPT	3OHPT
Mean (n=9) (%)	104	96.0	96.3
RSD (%)	2.7	2.3	4.1

Mean and RSD values for between run (n=9) results.

results are proprietary, only the SC and QC samples are used here to demonstrate long-term performance. Twenty-one analytical runs measuring PT in dog plasma over the period July 2001 to March 2002 were examined retrospectively for accuracy and precision of SC and QC samples. Each run consisted of system suitability check samples, reconstitution blank samples, a set of SC samples over the range 0.117–117 n*M* (0.1–100 ng/ml), QC samples in triplicate at 0.117, 0.351, 35.1 and 117 n*M* (0.1, 0.3, 30 and 100 ng/ml), and typically 228 analytical samples. The mean accuracy and RSD values for the SC and QC samples in the runs are shown in Table 7. The SC accuracy varied from 98.1 to 103% and the RSD varied from 1.8 to 6.1% (n=21 at each level). The QC accuracy varied from 97.7 to 98.9% and the RSD varied from 5.5 to 11 % (n=63 at each level). No SC samples were excluded in any runs. Of the 252 QC samples only six were outside the acceptance limits and no more than one per assay run. The above results show that the assay is accurate and precise for large sample runs over an extended period of time.

Table 7
Mean Paclitaxel SC and QC accuracy and precision over 21 analytical runs of dog plasma samples

Sample	Accuracy (%)	RSD (%)	*n*
SC-0.1	100	1.8	21
SC-0.3	100	6.1	21
SC-0.75	98.8	4.0	21
SC-1	100	3.2	21
SC-3	103	4.1	21
SC-7.5	98.1	2.8	21
SC-10	98.1	2.4	21
SC-30	102	2.7	21
SC-75	99.1	3.5	21
SC-100	101	4.7	21
QC-0.1	97.7	11	63
QC-0.3	98.9	11	63
QC-30	98.0	5.5	63
QC-100	98.1	6.5	63

4. Conclusions

The development and validation of this method illustrate the importance of and interdependence between the chromatographic and mass spectrometric elements of an LC–MS assay. Preliminary decisions based upon the assay goals and consideration of GLP recommendations guided the development strategy. Determination of the inherent sensitivity of MS to the target analyte identified the minimum amount of analyte necessary to give a reproducible signal. A clean and efficient sample extraction permitted the use of a short, narrow-bore column. This in turn lowered the detectable plasma concentration for a given instrument sensitivity and shortened the run time. Minimizing the sample size meant that the column and instrument remained cleaner, allowing longer runs and requiring less instrument downtime for cleaning and recalibration.

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