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Quantitative determination of paclitaxel in human plasma using semi-automated liquid–liquid extraction in conjunction with liquid chromatography/tandem mass spectrometry

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Abstract

This paper describes a high-throughput sample preparation procedure combined with LC–MS/MS analysis to measure paclitaxel in human plasma. Paclitaxel and an internal standard were extracted from plasma by a semi-automated robotic method using liquid–liquid extraction. Thereafter compounds were separated on a RP C18 column. Detection was by a PE Sciex API 3000 mass spectrometer equipped with a TurboIonSpray™ interface. The compounds were detected in positive ion mode using the mass transition m/z 854.6 → 286.2 and m/z 831.6 → 263.2 for paclitaxel and the internal standard, respectively. The limit of quantitation for paclitaxel was 1 ng/ml with an imprecision of 5.2% following extraction of 0.1 ml of plasma. Linearity was confirmed over the whole calibration range (1–1000 ng/ml) with correlation coefficients higher than 0.99 indicating good fits of the regression models. The inter and intra-day precision was better than 9.5% and the accuracy ranged from 90.3 to 104.4%. The assay was simple, fast, specific and exhibited excellent ruggedness.

Keywords: Paclitaxel; Semi-automated liquid–liquid extraction; LC–MS; Human plasma

1. Introduction

Paclitaxel is one of the most important and promising anticancer agents introduced during the past two decades. The antineoplastic activity of paclitaxel is known to be mediated by binding to tubulin, stabilising microtubules and blocking the transit of cell cycling from G2-phase to the M-phase [1,2]. Currently, paclitaxel is indicated in the treatment of breast cancer after failure of combination chemotherapy, ovarian cancer refractory to cisplatin-based regimens, non-small cell lung cancers and melanoma [3–5]. A number of assay methods have been published for the determination of paclitaxel in biological fluids, including capillary electrophoresis [6], liquid chromatography-mass spectrometry [7–9], immunoassay [10,11] and HPLC [12–30]. These methods utilise either solid-phase extraction (SPE) [12–17], on-

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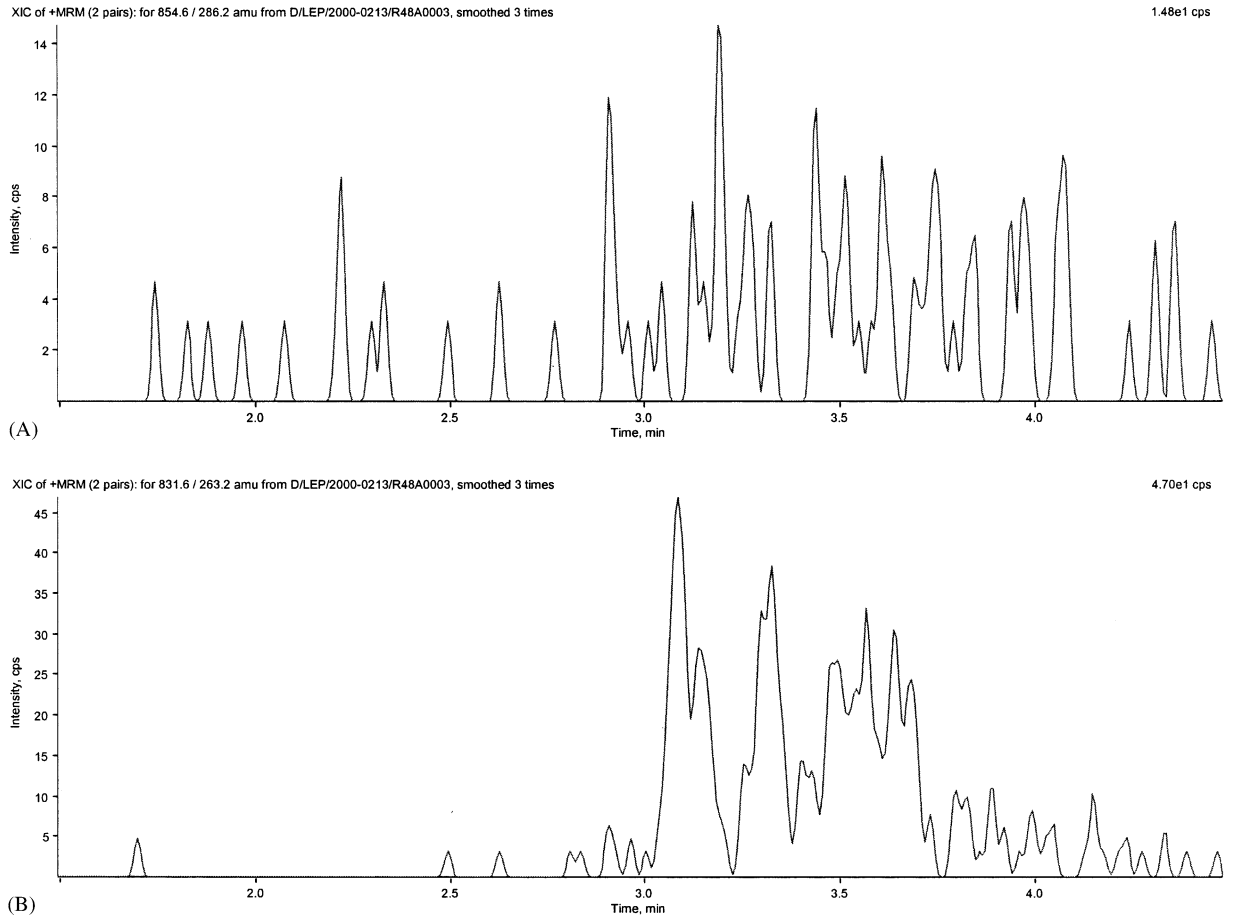

Fig. 1. MRM chromatograms resulting from the analysis of blank (paclitaxel and internal standard free) human plasma extract. Panels A and B correspond to the MRM channels for paclitaxel and the internal standard, respectively.

line SPE [9], protein precipitation and SPE [18], liquid–liquid extraction (LLE) [12,19–22], LLE using micro sample volumes [23], solvent extraction followed by column-switching [24], or SPE followed by column-switching [25], or solvent extraction and SPE [26–30].

Recently, several laboratories have demonstrated the utility of the high-throughput LLE extraction approach to improve efficiency of sample processing [31–33].

Given the current shift towards automated and robotics system, we explored the utility of a LLE procedure as an alternative to previously reported manual sample preparation protocols for analysis of paclitaxel.

2. Experimental

2.1. Materials

Paclitaxel (Fig. 3A) was supplied by Sigma–Aldrich (Milan, Italy) and the analogue internal standard (IS, Fig. 3B) was supplied by Pharmacia Italia S.p.A. Blank human plasma was obtained from Bio-Media (Boussens, France). All other

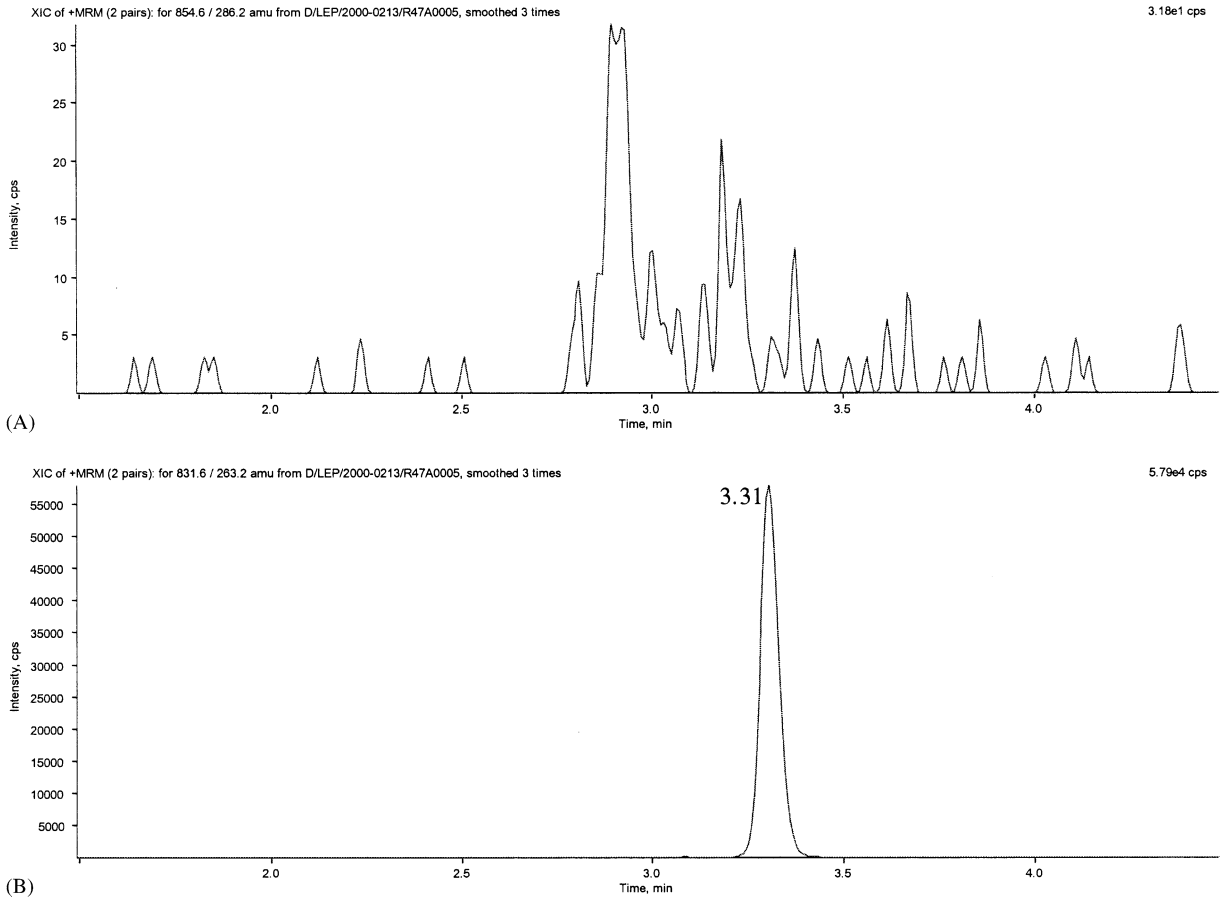

Fig. 2. MRM chromatograms resulting from the analysis of paclitaxel free (blank) human plasma (panel A) spiked with the internal standard (panel B).

chemicals were commercially available and of analytical grade.

2.2. Chromatographic conditions

The liquid chromatography (LC) system consisted of a HP1100 system (Agilent, Cernusco s/n, Italy) connected with an autosampler AS200 (Perkin–Elmer Italia, Monza, Italy) equipped with a 96-well plate holder. A 150 × 4.6 mm i.d., 5 μm, SB C18 Zorbax column (CPS Analitica, Milan, Italy) was utilised for chromatographic separation. The LC flow rate was 1 ml/min and was split so that 100 μl/min was directed towards the mass spectrometry (MS) interface. The compounds were eluted using a mobile phase of 65:35 acetonitrile:buffer (2 mM ammonium acetate, pH 5) held isocratically for 0.5 min, followed by a 0.5 min linear gradient to 98:2 (acetonitrile:buffer), and then by a 3.5 min isocratic elution before to return to the initial conditions in 0.5 min. After 1 min of re-equilibration, the column was ready for a new injection. Separation was performed at 45 °C.

2.3. MS–MS conditions

An API 3000 triple quadrupole mass spectrometry (Applera, Monza, Italy) with a TurboIonSpray™ interface in the positive ion mode was used for detection. The mass spectrometer was operated in the multiple reaction monitoring

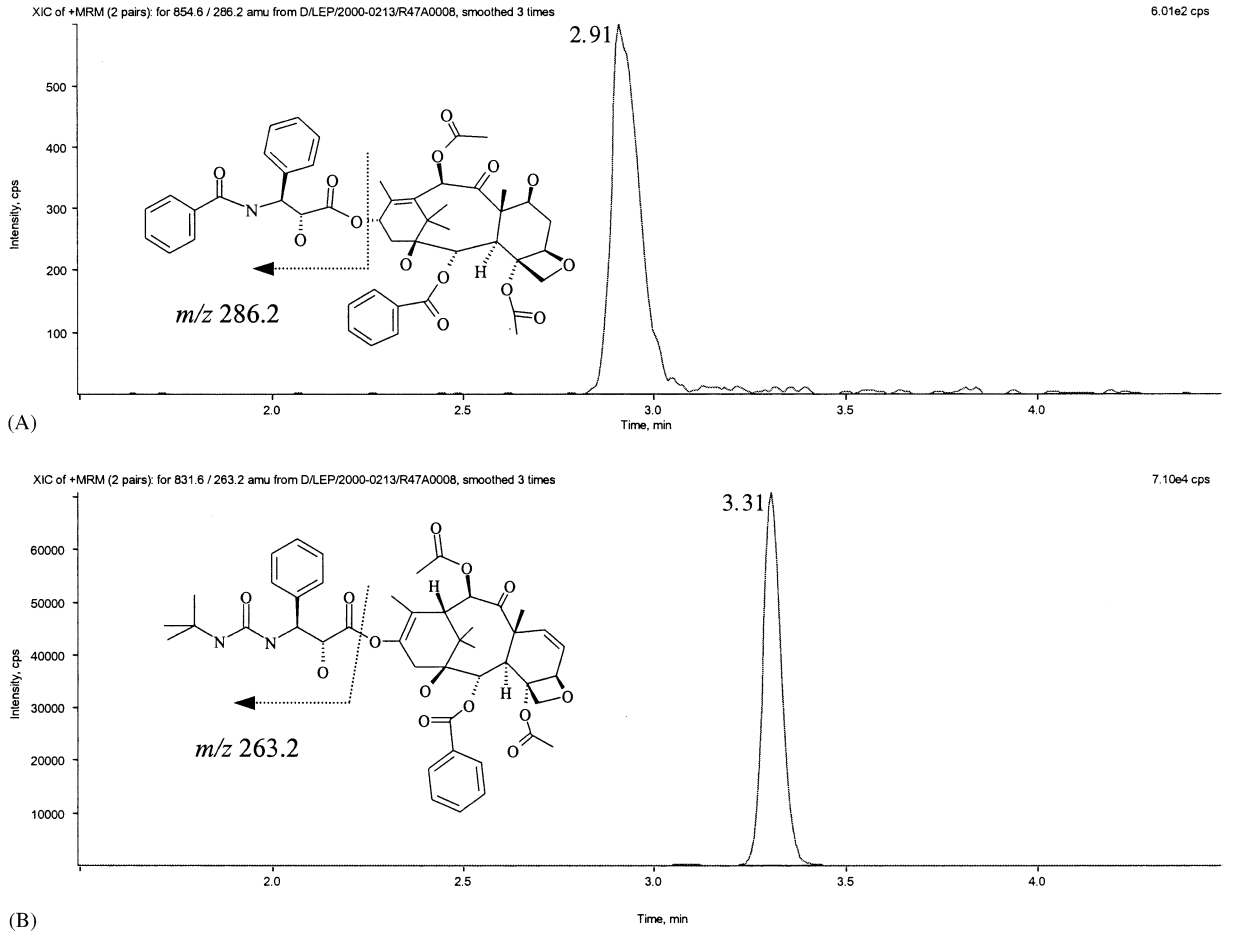

Fig. 3. MRM chromatograms resulting from the analysis of 0.996 ng/ml of paclitaxel and internal standard. Panels A and B correspond to paclitaxel (MRM transition: m/z 854.6→286.2) and the internal standard (MRM transition: m/z, 831.6→268.2), respectively. Molecular structures of both compounds are shown.

(MRM) mode. The TurboIonSpray™ source was operated at a temperature of 350 °C. Nitrogen was used as the curtain gas (setting 12), nebulizing gas (setting 5) and collision gas (setting 7). The collision energy (Q0-R02) was set at +25 V. The orifice (OR) and ring (RNG) voltages were set at 80 and 150 V, respectively. Following HPLC separation, the peak area corresponding to the m/z 854.6→286.2 reaction (dwell time 400 ms) for paclitaxel were measured relative to that of the m/z 831.6→263.2 reaction (dwell time 200 ms) of the internal standard. A Macintosh workstation was utilised for data acquisition and processing. Sample Control (version 1.4), Mac Quan (version 1.6) and Microsoft Excel (version 5.0) software were used for data processing and statistical analysis.

2.4. Preparation of solution, standards and QC plasma samples

Standard stock solutions of paclitaxel (one for calibrants and one for QC samples) were prepared by dissolving about 4 mg of the compound in 10 ml of ethanol. When stored at +4 °C these solutions were stable for at least 1 month. From the stock solution, working solutions were prepared each week in ethanol. Aliquots of these solutions were spiked into human plasma to

Table 1
Accuracy and precision of calibration curves for paclitaxel

Concentration added (ng/ml)	Concentration found (ng/ml)			Mean[a]	S.D.	%R.S.D.	%Bias
	Day 1	Day 2	Day 3				
0.996	1.130	1.042	1.101				
	0.864	0.934	0.892	0.994	0.112	11.3	−0.2
2.490	2.555	2.592	2.507				
	2.447	2.436	2.484	2.504	0.061	2.4	0.5
7.470	7.102	7.955	7.691				
	7.505	7.384	7.150	7.465	0.326	4.4	−0.1
24.91	24.97	25.36	25.62				
	24.68	24.78	23.57	24.83	0.712	2.9	−0.3
98.90	112.0	105.3	113.2				
	101.5	103.7	94.96	105.1	6.8	6.5	6.3
402.9	430.1	398.8	404.7				
	391.2	388.0	386.7	399.9	16.3	4.1	−0.7
989.0	894.5	986.3	1002				
	929.0	858.1	942.1	935.3	54.3	5.8	−5.4

[a] $n = 6$.

obtain final concentration of 0.996, 2.49, 7.47, 24.9, 98.9, 403 and 989 ng/ml. These samples were used to evaluate the linearity of the method.

For within-study assay validation QC samples were prepared by spiking blank human plasma with paclitaxel working solutions to yield the following concentrations 2.99, 399, 832 and 6930 ng/ml. The QCs were stored at −20 °C, pending analysis.

2.5. Plasma extraction procedure

The LLE method was partially automated using a Multiprobe II (Packard Bioscience, Pero, Italy) robot.

Eppendorf vials containing plasma samples were positioned in an adapted Multiprobe II rack. Aliquots (100 μl) of the plasma sample were transferred to a rack containing glass tubes. Aliquots (100 μl) of IS and (900 μl) of *tert*-butyl methyl ether (TBME) were then transferred into the tubes from the respective reservoirs. The glass tubes were then manually capped and shaken for 3 min followed by centrifugation at $1200 \times g$ for 3 min. The tubes were then returned to the Multiprobe II instrument and the organic layer (0.6 ml) for each sample was then transferred into a clean 96-well plate. The organic solvent was dried, by passing nitrogen gas to each of the wells at 25 °C, using a 96-well drying block. Reconstitution of sample residues was performed by adding 100 μl of 2 mM ammonium acetate buffer solution, pH 5: acetonitrile mixture (75:25, v/v), using the Multiprobe II. The 96-well plate was sealed with a cover mat (Capmats, silicon rubber, Whatman Inc. Clifton, NJ, USA) and, after vortex mixing, was centrifuged at $2000 \times g$ for 3 min at 4 °C and placed on the autosampler followed by injection of a 25 μl portion into the LC column for analysis.

2.6. Calculations

The calibration curve was plotted using weighted linear least-squares regression analysis (weighting factor $1/x^2$) according to the equation $y = a + bx$, where y is the peak-area ratio, x is the concentration of the calibration samples, "a" is the intercept and "b" is the slope of the regression line. The weighting factor was chosen to minimise deviation of back-calculated values from theoretical concentrations. Subsequently, concentrations of the quality control (QC) samples were calculated from the regression equation of the calibration curve.

Table 2
Accuracy and precision of the QC sample for paclitaxel

	Concentration added (ng/ml)	Accuracy			Inter-day		
		Day 1	Day 2	Day 3	Mean	S.D.	%R.S.D.
	2.989	91.3	102.6	88.0	93.9	5.4	5.8
		92.2	98.7	94.4			
		84.2	99.5	89.9			
		96.5	101.5	88.1			
		92.8	98.3	91.0			
Mean		91.4	100.1	90.3			
S.D.		4.5	1.9	2.6			
%R.S.D.		4.9	1.8	2.9			
	398.5	98.5	99.8	100.4	100.3	8.2	8.2
		109.6	92.8	94.0			
		102.7	110.9	90.4			
		96.9	113.7	87.1			
		92.7	104.9	110.3			
Mean		100.1	104.4	96.4			
S.D.		6.4	8.4	9.2			
%R.S.D.		6.4	8.1	9.5			
	831.6	93.0	108.2	99.4	98.7	6.6	6.7
		105.1	99.2	93.4			
		96.3	110.1	89.8			
		91.1	106.9	93.7			
		98.1	92.8	103.1			
Mean		96.7	103.4	95.9			
S.D.		5.4	7.3	5.3			
%R.S.D.		5.6	7.0	5.5			
	6930[a]	95.4					
		96.3					
		96.7					
		100.5					
		99.1					
Mean		97.6					
S.D.		2.1					
%R.S.D.		2.2					

$N = 5$ for intra-day measurements. $N = 15$ for inter-day measurements. Accuracy = [observed value/nominal value] × 100.
[a] QC samples out of calibration range evaluated after diluting 1 to 50 with blank human plasma.

3. Results and discussion

A Q1 scan of paclitaxel and IS revealed the protonated parent molecule $(M+H)^+$ to be in abundance with a mass to charge ratio (m/z) of 854.6 and 831.6, respectively. The product ion spectrum using collision energy of 25 eV resulted in a major fragment at m/z 286.2 for paclitaxel and m/z 263.2 for IS. Proposed structures for these fragment ions are shown in Fig. 3.

Under the chromatographic conditions utilised in this study, the retention time was about 2.9 and 3.3 min ($k' = 0.93$ and 1.2) for paclitaxel and the IS, respectively. The use of a gradient allowed separation of paclitaxel and IS with good resolution, maintaining good peak shapes for both compounds. The re-equilibration time of the column (1 min) at the end of the gradient, followed by the time (about 1 min) required by the autosampler to wash the system and inject a new

Table 3
Stability of paclitaxel in human plasma

Storage conditions	Concentration (ng/ml)	Accuracy
24 h at room temperature	2.989	89.1
	398.5	91.4
	831.6	87.3
48 h at room temperature after extraction	2.989	100.1
	398.5	107.6
	831.6	100.8
After three freeze/thaw cycles	2.989	99.3
	398.5	100.5
	831.6	103.1

$N = 3$ for each concentration measurement. Accuracy = [Observed value/nominal value] × 100.

sample, was sufficient to fully recondition the column and good reproducibility of the retention times was obtained. The R.S.D. of the retention time after more than 60 injections was less than 0.5% for both compounds.

During the elution of both compounds, the high concentration of organic phase (98%) coupled with the temperature of the TurboIonSpray interface improved effluent evaporation, thereby decreasing the back-ground and increasing the ionisation efficiency and sensitivity. The selectivity of the method was examined by analysing blank human plasma extracts with and without the internal standard. As shown in Figs. 1 and 2 no interference in the blank plasma trace was seen from endogenous substances. Fig. 3 depicts a representative time–intensity plot for the lower limit of quantitation (LLOQ, 0.996 ng/ml) of the calibration curve. Good sensitivity was observed following injection of 25 μl of the final extract, corresponding to 17 pg of paclitaxel on column with a S/N higher than 10. The precision and accuracy at this LLOQ concentration level, assessed by analysis of six spiked human plasma samples in one analytical run, was 5.2% (R.S.D.) and 94.2%, respectively.

Tables 1 and 2 show validation data obtained during 3 days of analysis. Table 1 shows the calibration curve parameters for paclitaxel. Standard samples were prepared in duplicate and analysed to generate a calibration curve on each validation day. The calibration curves were linear over the range 1–1000 ng/ml. The coefficients of linear regression were higher than 0.99. Back calculated values for the calibration points showed a %R.S.D. lower than 11.3 and a %Bias ranging from −5.4 to 6.3. Five replicates of the QC samples at three concentration levels were analysed on each validation day. Table 2 summarises the intra and inter-assay accuracy and precision of the QC samples for paclitaxel. Parallelism was assessed by analysis of "over-range" paclitaxel QC samples. After a 50-fold dilution with control human plasma, the samples demonstrated a mean accuracy of 97.6 and a %R.S.D. of 2.2 (Table 2).

The results of paclitaxel stability assessment in human plasma are reported in Table 3. There was no evidence of degradation of paclitaxel after storage in plasma at room temperature for 24 h, during storage after extraction at room temperature for 48 h and after three freeze/thaw cycles. Mean response values for stored samples were within ±20% of responses for fresh samples, under all storage conditions examined. The recovery of paclitaxel from spiked plasma samples ranged from 70 to 86% when assessed by comparing the

Table 4
Recovery of paclitaxel from human plasma

	Concentration (ng/ml)	Recovery (%)	R.S.D. (%)	*N*
Plasma added with paclitaxel	2.989	71.5	6.2	3
	398.5	81.9	6.7	3
	831.6	86.2	8.0	3
Plasma added with ^{14}C-paclitaxel	400	92.0	1.1	8
	800	91.5	2.4	8

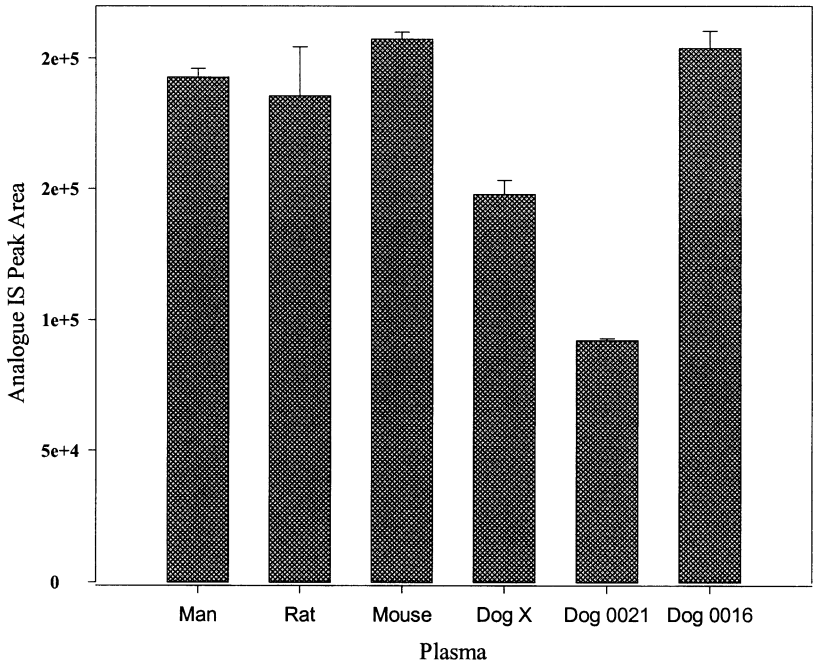

Fig. 4. Plasma effect in quantitation of analogue IS (mean ± S.D., $n = 3$).

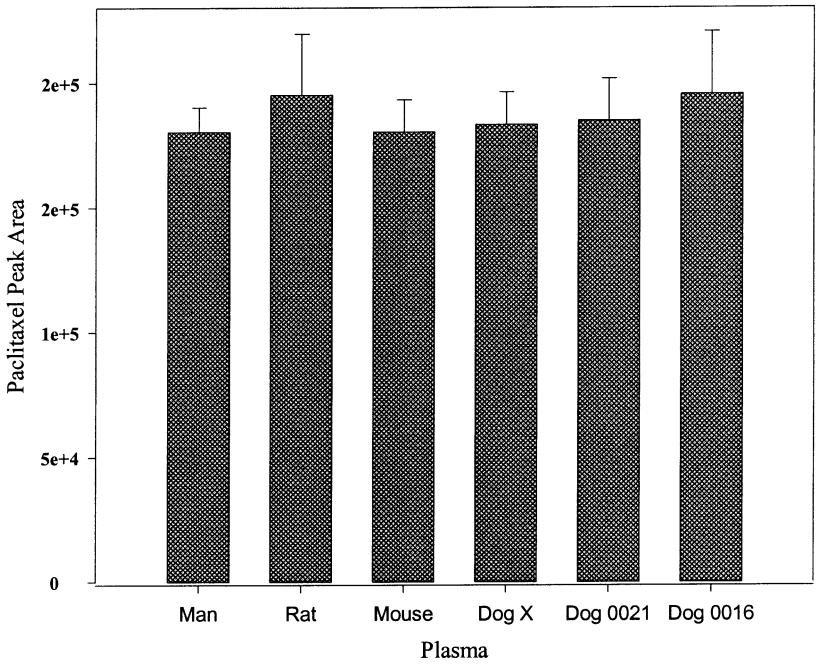

Fig. 5. Plasma effect in quantitation of paclitaxel (mean ± S.D., $n = 3$).

peak area of the analyte obtained from extracted samples and those obtained after injection of the standard solution. This finding was confirmed in a further study where human plasma samples were spiked with ^{14}C-paclitaxel and the radioactivity was evaluated using beta-counter instrument (Table 4). To increase the sample preparation speed, use of protein precipitation with methanol or acetonitrile was tested but very low and variable recoveries were obtained. As previously reported [23] and also as described in this paper, using TBME good recovery of paclitaxel was obtained. This extraction recovery was greater than that described in several previously published methods that have utilised various extraction techniques [12–15,20,24].

The semi-automated sample preparation procedure is rapid, however, the use of appropriate 96-well block instead of glass tubes could further enhance the speed of the sample handling and processing procedure. We attempted to use standard round and square 96-well blocks with different types of caps but in all cases we obtained cross-contamination in the samples after agitation of the block containing TBME. Subsequent to the validation experiment reported in this paper, it was found that using 96-polypropylene tube blocks from Micronics systems (Micronics B.V., Lelystad, The Netherlands) prevented cross-contamination as the 96-well is constructed from a separate unit for each well.

This methodology was extended to the determination of paclitaxel in plasma from different animal species as well as human. However, a variable response for the internal standard was sometimes observed. Detailed comparison of the instrumental response (peak area) obtained from standard solutions and standard solutions added to plasma extracts indicated an ion suppression effect on the internal standard response in dog plasma in comparison with other species; differences were also observed between plasma from different dogs (Fig. 4). The same effect was not evident for paclitaxel in human plasma and other species tested (Fig. 5). Whilst the validity of this method (using an analogue of paclitaxel as IS) for paclitaxel was confirmed, this experiment demonstrated the additional potential benefits of a stable label IS and provides an example of the potential risk of ion suppression effects when changing matrices for a LC–MS method. Further development of this method to include a stable labelled internal standard (^{13}C-paclitaxel) will be reported in future.

4. Conclusions

In conclusion the reported semi-automated LLE method presents a simple and attractive alternative to previous manual LLE procedures. The method also demonstrates the general utility and efficiency of automated LLE procedures, which could be further considered for other analytes instead of SPE or protein precipitation. Ion suppression experiments with plasma of different species indicate that in some circumstances the structural analogue internal standard may suffer an ion suppression effect not seen by the parent compound. Whilst there is no indication this is a major problem for the analysis of paclitaxel in human plasma, the method will be further improved in the future by the inclusion of a stable label internal standard.

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