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Ultra-sensitive assay for paclitaxel in intracellular compartments of A549 cells using liquid chromatography–tandem mass spectrometry

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ABSTRACT

A high-performance liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the determination of paclitaxel in intracellular compartments using docetaxel as internal standard (IS) has been developed and validated. A549 cancer cells (10^6) were incubated with paclitaxel (2 ng/mL) for up to 4 h and then subjected to sequential extraction of cytosolic, membrane/organelle, nuclear and cytoskeleton soluble protein. Fractions were ultrasonicated to release protein bound paclitaxel after which drug was extracted using liquid–liquid extraction with diethyl ether:dichloromethane (2:1, v/v). Chromatographic separation was then carried out on an Ascentis Express C18 column (50 mm × 4.6 mm, 2.7 μm) with a mobile phase of acetonitrile:0.1% formic acid in water (50:50, v/v). Detection involved electrospray positive ionization followed by multiple reactions monitoring of the precursor-to-product ion transitions of paclitaxel at *m/z* 854.4 → 286.3 and docetaxel at *m/z* 808.6 → 226.1. Assay validation based on samples of total cell extract in the same buffer as protein fractions showed the assay was linear over the range 2–600 pg/mL with intra- and inter-day precision (as relative standard deviation) and accuracy (as relative error) of <7% and <±12%, respectively. Recovery was approximately 70% and matrix effects were minimal. The distribution of paclitaxel in subcellular components of A549 cancer cells was mainly into the cytoskeletal compartment.

1. Introduction

Paclitaxel is a mitotic inhibitor widely used in the treatment of ovarian cancer, breast cancer, non small-cell lung cancer, prostate cancer and melanoma [1–3]. It works by binding to tubulins, the proteins that make up the microtubules of the cellular cytoskeleton [4]. Microtubules are attractive and established targets of many anticancer agents, which cause strong cytotoxicity by disrupting cell division leading to apopotic, cell death [5]. Unlike other tubulin-targeting drugs such as colchicines, which inhibit microtubule assembly, paclitaxel stabilizes the microtubule polymer and protects it from disassembly. Together with docetaxel it forms the drug category known as the taxanes.

Paclitaxel acts intracellularly and exhibits nonlinear pharmacokinetics with saturable distribution to the tissues [6,7]. Thus, determination of plasma concentrations of paclitaxel do not accurately reflect the concentration of drug in target tissues. In addition, the concentration of paclitaxel in target tissues and the intracellular concentration do not correlate. Since the subcellular distribution of paclitaxel may reflect its ability to bind to microtubules, it is important to investigate its distribution at the subcellular level.

Numerous analytical methods have been applied to the determination of paclitaxel in biological fluids. High-performance liquid chromatography with ultraviolet detection (HPLC–UV) has often been used but suffers from low sensitivity due to the weak UV absorbance of paclitaxel [8–10]. In recent years, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been used to determine paclitaxel in human, dog, mouse and rat plasma as well as in rat tissues. Thus Tong et al. used LC–MS/MS to quantify paclitaxel in rat tissue homogenates with a lower limit of quantitation (LLOQ) of 0.2 ng/mL [11]. Gaspar et al. achieved a

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much lower LLOQ of 20 pg/mL in tumor cells using a selective solid phase extraction (SPE) method and capillary LC–MS/MS [12]. Yu et al. using a similar methodology were able to analyze paclitaxel in cancer cells with an LLOQ of only 5 pg/mL [13]. However, to date there are no reports of the subcellular quantitation of paclitaxel.

Quantitation of paclitaxel at the subcellular level is challenging because of its low concentration in intracellular compartments after administration of therapeutically relevant doses [11–18]. In the past, commonly used methods to study anticancer drug binding to microtubules included confocal microscopy and radioactive analysis [19,20]. Although the latter provides high sensitivity, it has inherent disadvantages such as the need for expensive radiolabeled drug, the fact that it may disturb the normal physiological state of the cell and its inability to differentiate parent drug from metabolites. LC–MS/MS has none of these disadvantages and provides high selectivity and sensitivity [4,21]. In this study, we applied LC–MS/MS to determine paclitaxel in intracellular compartments of A549 non-small cell lung cancer cells after incubation with non-cytotoxic concentrations of the drug.

2. Experimental

2.1. Chemicals and reagents

Paclitaxel (>99.0% purity) was purchased from the National Institute for Food and Drug Control (Beijing, PR China). Docetaxel and HPLC grade acetonitrile were obtained from Sigma–Aldrich (St. Louis, MO, USA). Formic acid and sodium hydroxide were obtained from Beijing Chemical Plant (Beijing, PR China) and HPLC grade methanol was from Fisher Scientific (Fair Lawn, NJ, USA). Ultra-high purity water, prepared using the Milli-Q system, was used throughout the study. A549 cells were purchased from American Type Culture Collection (Rockville, MD, USA) Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Invitrogen, Grand Island, NY). The ProteoExtract™ Subcellular Proteome Extraction Kit was purchased from Merck KGaA, Darmstadt, Germany.

2.2. Preparation of calibration standards and QC samples

A stock solution (1 mg/mL) of paclitaxel in methanol was diluted with methanol:water (50:50, v/v) to produce standard solutions with concentrations of 0.1, 0.3, 1, 3, 10, and 30 ng/mL. Quality control (QC) solutions were prepared independently at concentrations of 0.3, 3 and 24 ng/mL in the same way. An aliquot (50 μL) of each solution was mixed with 2450 μL total cell lysate (see Section 2.4) to produce calibration standards with concentrations of 2, 6, 20, 60, 200 and 600 pg/mL and low, medium and high QC samples with concentrations of 6, 60 and 480 pg/mL. A stock solution (1 mg/mL) of the internal standard (IS), docetaxel, in methanol was diluted in methanol:water (50:50, v/v) to give a 2 ng/mL working solution. All solutions were stored at 4 °C when not in use.

2.3. LC–MS/MS conditions

Chromatography was performed using a Shimadzu LC-20ADXR HPLC system (Shimadzu, Kyoto, Japan) equipped with an Ascentis Express C18 column (50 mm × 4.6 mm, 2.7 μm) maintained at 40 °C. The mobile phase consisted of acetonitrile:0.1% formic acid in water (50:50, v/v) delivered at a flow rate of 0.8 mL/min. Mass spectrometric detection employed a Qtrap 5500 mass spectrometer (AB Sciex, Ont., Canada) equipped with an electrospray ionization (ESI) source operated in the positive ion mode. The curtain gas, Gas 1 and Gas 2 were nitrogen set at 30, 20, and 30 psi, respectively. The ion spray voltage was adjusted to 5500 V and the source temperature was set at 450 °C. Detection was by multiple reaction monitoring (MRM) at low resolution for Q1 and unit resolution for Q3 with a dwell time of 200 ms per channel. Paclitaxel and IS were monitored using the transitions of the protonated molecular ions to their most intense product ions at m/z 854.4 → 286.3 and m/z 808.6 → 226.1 respectively, with declustering potential 30 V and collision energy 26 eV. Mass spectrometric parameters were tuned to maximize the response of the precursor/product ion combinations. Data acquisition and integration were controlled by Applied Biosystems Analyst version 1.5 software.

2.4. Preparation of subcellular fractions and total cell lysate

The ProteoExtract™ Subcellular Proteome Extraction Kit is designed for sequential extraction of protein fractions from cells based on differences in protein solubility of subcellular fractions (cytosolic, membrane/organelle, nuclear and cytoskeleton). The cells are plated and cells or parts of cells remain attached to plates during sequential extraction to prevent mixing of the four subcellular compartments. A549 cells (1×10^6 cells) were cultured in 2 mL DMEM in fourteen 6 cm diameter Petri dishes for 2 days after which the medium was replaced with 2 mL fresh DMEM containing 2 ng/mL paclitaxel. Plates were incubated and two plates removed at 0.5, 1, 1.5, 2, 2.5, 3 and 4 h. Cellular fractions of soluble protein were then prepared using the ProteoExtract™ kit according to the manufacturer's instructions [24]. This involved (1) preparation of cytosolic fraction by incubating cells with 200 μL Extraction Buffer I for 10 min at 4 °C, washing with 2 mL Wash Buffer and transferring the Wash Buffer (cytosolic fraction) to an Eppendorff tube; (2) extraction of the remaining protein by incubating sequentially at 4 °C with 200 μL Extraction Buffers II–IV for 30 min, 10 min and 5 min respectively and treating as in (1) to give membrane/organelle, nuclear and cytoskeletal fractions respectively each in 2 mL Wash Buffer. All fractions were then subjected to ultrasonication (4×30 s on half-power) to disrupt paclitaxel protein binding followed by centrifugation at 9000 × g, 4 °C for 20 min. Protein was determined in fractions using the BCA Protein Assay Kit. Total cell lysate for use in assay validation was prepared by culturing A549 cells (10^6) in 2 mL DMEM for 2 days, replacing DMEM with 2 mL Wash Buffer, suspending the cells using a cell scraper and finally ultrasonicating and centrifuging as described above. Subcellular fractions and total cell lysate were stored at −20 °C pending analysis by LC–MS/MS.

2.5. Sample preparation

To 500 μL subcellular fraction or calibration standard or QC sample in a glass tube, 100 μL IS working solution and 100 μL methanol:water (50:50) were added. The mixture was then shaken with 3.5 mL diethyl ether:dichloromethane (2/1, v/v), centrifuged for 5 min at 3500 × g and the organic layer transferred to another glass tube and evaporated to dryness at 40 °C under nitrogen. The residue was reconstituted in 100 μL mobile phase and, after centrifugation for 5 min at 9000 × g, the supernatant (40 μL) was injected into the LC–MS system.

2.6. Assay validation

Assay validation was performed according to the guidance of the US Food and Drug Administration [22,23]. Linearity was assessed by weighted ($1/x^2$) least-squares linear regression of calibration curves based on peak area ratios prepared in triplicate on three separate days. Precision (as relative standard deviation, RSD) and accuracy (as relative error, RE) were evaluated based on assay of six replicates of QC samples on three different days. The LLOQ was defined as the lowest concentration with precision <15% and accuracy of ±15%. Selectivity was investigated by analyzing six replicates of total cell lysate spiked at the LLOQ. Recovery was

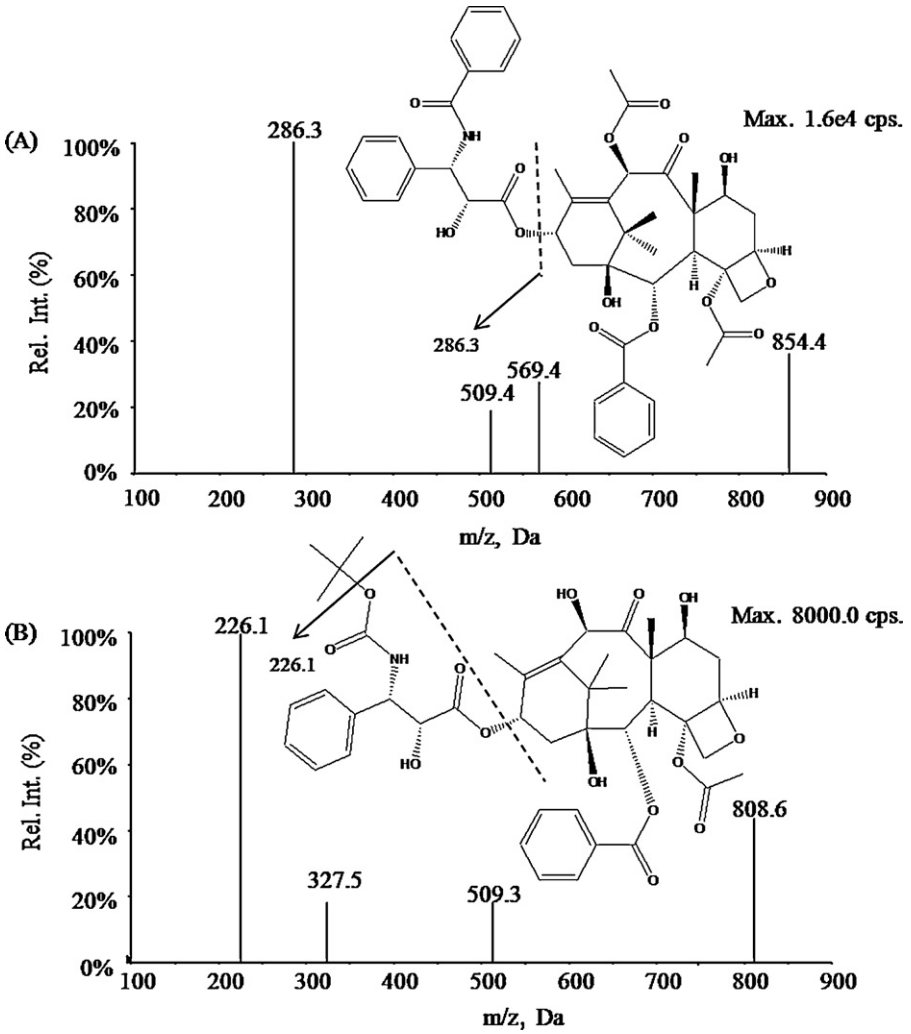

Fig. 1. Structures, proposed fragmentation patterns and full-scan product ion spectra of (A) paclitaxel and (B) docetaxel.

calculated by comparing peak areas of QC samples with those prepared by spiking the upper organic layer of extracted total cell lysates with the corresponding concentrations. Matrix effects were determined by comparing the peak areas of six replicate total cell lysates spiked after centrifugation at 6, 60 and 480 pg/mL with the mean peak areas of corresponding standard solutions prepared in the same way but substituting water for total cell lysate. The stability of paclitaxel in total cell lysate was investigated by analysis of triplicate QC samples stored at 25 °C for 12 h, at −20 °C for 1 month and after three freeze-thaw cycles (−20 °C to room temperature). Stability of extracted samples stored at room temperature for 12 h was also assessed.

3. Results and discussion

3.1. LC–MS/MS conditions

Both analyte and IS responded best to positive ionization and gave protonated $[M+H]^+$ ions as major peaks. The chemical structures of paclitaxel and docetaxel with proposed fragmentation patterns based on the full-scan product ion spectra of $[M+H]^+$ ions are shown in Fig. 1. Quantitation was based on peak area ratios for transitions from $[M+H]^+$ to the most abundant product ions of analyte and IS. In terms of the chromatography, evaluation of various mobile phases containing different combinations of acetonitrile, methanol, 10 mM ammonium acetate and water showed that inclusion of acetonitrile produced strong signals with no solvent-clustered ions and use of 0.1% aqueous formic acid instead of water improved peak shape. Due to the poor solubility of paclitaxel, its elution required a relatively large portion of acetonitrile in the mobile phase, which, at a flow rate of 0.8 mL/min, allowed an analytical run time of only 5 min. The fused core particles in the Ascentis Express column provide high column efficiency due to their small partiele size (2.7 μm) and the shallow diffusion path of their 0.5 μm thick porous shell.

3.2. Sample preparation

As paclitaxel is highly lipophilic and only weakly polar, liquid–liquid extraction (LLE) was expected to provide good recovery. On comparing methyl tert-butyl ether, n-hexane and mixtures of diethyl ether and dichloromethane as extraction solvents, it was found that, at low paclitaxel concentrations, recovery was very sensitive to the nature of the solvent and was improved by adding methanol:water (50:50, v/v) and using a mixture of diethyl ether and dichloromethane. Through evaluation of different (v/v) combinations of these solvents, it was found that 3:2 gave low recovery, 4:1 produced high but variable recovery for different concentrations of paclitaxel and 2:1 gave efficient extraction of both paclitaxel and IS with minimum interference. To reduce matrix effects, it was found necessary to ultrasonicate cells both before

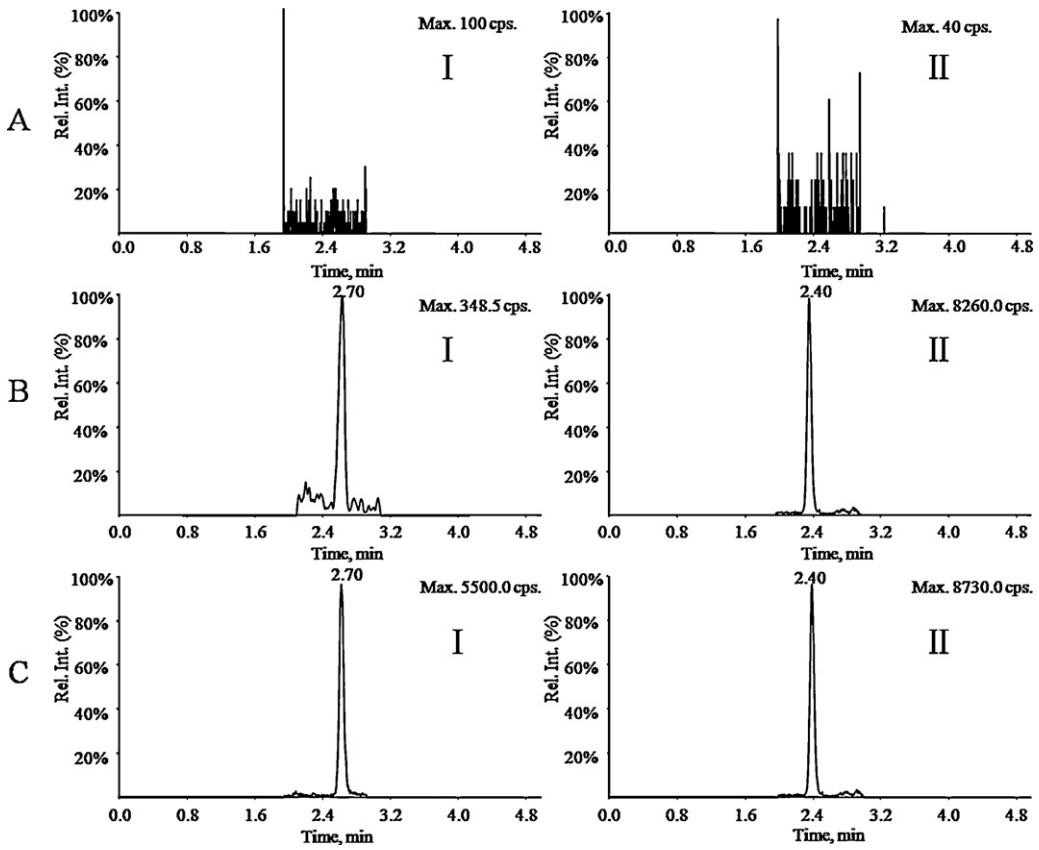

Fig. 2. Representative LC–MS/MS chromatograms for (I) paclitaxel and (II) docetaxel in (A) A549 total cell lysate, (B) total cell lysate spiked with paclitaxel at the LLOQ (2 pg/mL) and IS (2 ng/mL) and (C) a sample containing the cytoskeletal fraction from A549 cells after incubation with 2 ng/mL paclitaxel for 1.0 h (paclitaxel conc. 11.3 pg/mL).

and after LLE. Using this method of sample preparation, the assay was essentially free of matrix effects.

3.3. Assay validation

As shown in Fig. 2A, no significant interference was observed from endogenous substances in total cell lysate at the retention times of paclitaxel and IS. Fig. 2 shows a representative ion chromatogram of a standard sample at the LLOQ (2 pg/mL) and of a sample derived from cells incubated with paclitaxel for 0.5 h. The method was linear over the concentration range 2–600 pg/mL with a typical equation of $y = 0.00307x + 0.0248$, $r = 0.9974$. Intra- and inter-day precisions were <7% and <12%, respectively with accuracy <±5% for both (Table 1). Recoveries (mean ± S.D.%) of paclitaxel for low, medium and high QC samples were 67.6 ± 5.7%, 76.2 ± 9.6% and 77.4 ± 4.5% respectively. In terms of matrix effects, the ratios of the peak responses for low, medium and high QC samples were 92.4 ± 7.8%, 91.2 ± 7.0% and 97.9 ± 4.7%, respectively. The ratio for the IS was 90.9 ± 5.6%. Results of stability tests (Table 2) showed paclitaxel was stable under all conditions investigated (Table 3).

Table 1
Precision and accuracy for the determination of paclitaxel in A549 total cell lysate (data are means for assay of three replicates on three different days).

Concentration (pg/mL)		RSD (%)		RE (%)
Nominal	Found	Intra-day	Inter-day	
6.00	6.24	4.2	7.9	4.1
60.0	59.5	6.5	8.0	−0.8
480	494	6.0	11.2	2.9

3.4. Distribution in subcellular fractions and effect on tubulin dynamics

Assay of the four subcellular fractions showed that the distribution of paclitaxel was dependent on the incubation time. As shown in Fig. 3, after incubation for 0.5 h, paclitaxel was mainly present in the cytosol, nuclear and cell membrane whereas after 2.5 h, it was mainly localized in the cytoskeletal compartment. Antimitotic agents are known to stabilize microtubules by reducing the solubility of tubulin followed by redistribution of protein from the cytosolic (soluble) to the cytoskeletal (insoluble) fraction [24]. Since tubulins are dynamic structural components of the cellular cytoskeleton, the amount of paclitaxel associated with the

Table 2
Stability of paclitaxel in Wash Buffer stored under various conditions (data are means ±S.D. for assay of three samples at each concentration).

Conditions	Concentration (pg/mL)		RSD (%)
	Nominal	Found	
Long-term storage at −20 °C for 1 month	6.00	6.28 ± 0.62	9.8
	60.0	56.3 ± 6.0	10.7
	480	449 ± 34	7.5
Short-term storage at 25 °C for 12 h	6.00	6.04 ± 0.17	2.9
	60.0	60.8 ± 2.8	4.7
	480	471 ± 24	5.2
Three freeze–thaw cycles	6.00	6.12 ± 0.28	4.6
	60.0	56.5 ± 1.7	3.0
	480	445 ± 20	5.0
Processed samples at room temperature for 4 h	6.00	5.71 ± 0.18	3.2
	60.0	57.0 ± 2.3	4.0
	480	441 ± 10	2.3

Table 3
Paclitaxel and protein concentration in Wash Buffer containing intracellular fractions of A549 cells incubated with paclitaxel (2 ng/mL) for up to 4 h (data are means for assay of three samples of each cellular fraction).

Incubation time (h)	Cytosol		Membrane/organelle		Nucleus		Cytoskeleton	
	Paclitaxel (pg/mL)	Protein (mg/mL)	Paclitaxel (pg/mL)	Protein (mg/mL)	Paclitaxel (pg/mL)	Protein (mg/mL)	Paclitaxel (pg/mL)	Protein (mg/mL)
0.5	614	0.67	10,200	1.30	158	0.18	0.00	0.036
1.0	60.5	0.86	393	1.13	120	0.24	11.3	0.022
1.5	56.7	0.65	412	1.38	110	0.24	14.7	0.021
2.0	73.4	0.63	482	1.39	143	0.20	39.2	0.033
2.5	52.6	0.65	414	1.33	74.1	0.14	58.6	0.028
3.0	46.6	0.72	317	1.47	112	0.22	22.4	0.030
4.0	46.1	0.50	284	1.22	160	0.25	16.1	0.024

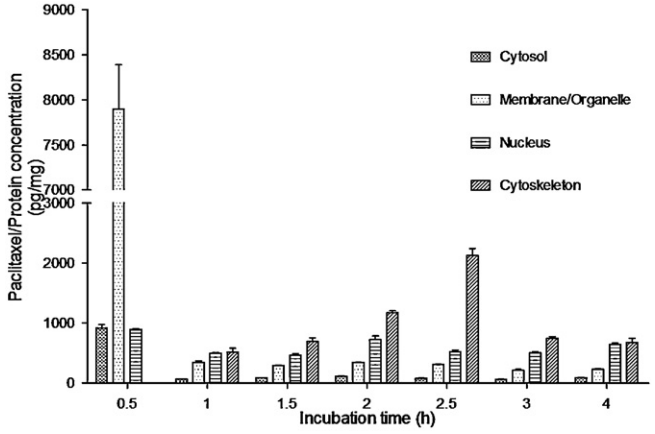

Fig. 3. The distribution of paclitaxel over time in the four subcellular fractions of A549 cells after incubation with 2 ng/mL paclitaxel. Data are mean ± S.D ($n = 3$).

polymerized microtubules in the cytoskeletal fraction reflects the effect of paclitaxel on tubulin polymerization. The localization in the cytoskeletal fraction after incubation for 0.5 h is consistent with previous results obtained using radiolabelled drug [24].

4. Conclusion

An ultra-sensitive LC–MS/MS method for the quantitation of paclitaxel in subcellular compartments of A549 cancer cells has been developed and validated. The method is suitable for investigating the efficacy of different formulations of paclitaxel in vitro.

Acknowledgments

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