Published in final edited form as:

J Pharm Biomed Anal. 2017 May 30; 139: 247–251. doi:10.1016/j.jpba.2017.03.011.

HPLC-MS/MS method for quantification of paclitaxel from keratin containing samples

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Abstract

Local drug delivery of paclitaxel is becoming ever more prevalent. As complex drug/excipient combinations are being developed and tested, new high performance liquid chromatography-mass spectrometry (HPLC-MS) techniques capable of quantifying paclitaxel from such formulations are needed. Here a method for quantifying paclitaxel from aqueous, protein and oil containing samples was developed and validated. Keratin, derived from human hair, is the protein component/paclitaxel excipient in the development and validation of said method. The novelty of this method is described by its ability to overcome water solubility issues and address clean-up of residual solvents in clinical grade paclitaxel injection composition. The method evaluates tert-butyl methyl ether and ethanol as extraction solvents with an extraction efficiency of $31.9 \pm 2.3\%$ and $86.4 \pm 4.5\%$ respectively. Upon evaporation and rehydration, samples were evaluated by HPLC-MS and a method was developed for paclitaxel quantification. The method developed had an inter-day precision of 9.1% relative standard deviation and an intra-day precision of 4.3% relative standard deviation normalized to a docetaxel internal standard. The described method is applicable to any aqueous paclitaxel sample containing protein and/or oils.

1. Introduction

Paclitaxel is an anti-proliferative drug extracted from pacific yew bark that inhibits cell division by binding to growing microtubules.^{1, 2} Used as a cancer therapeutic since 1992, paclitaxel is active against ovarian cancer, breast cancer, Kaposi's sarcoma and lung cancer.^{1, 3} More recently, paclitaxel has been used as an anti-restenotic in cardiovascular interventions such as drug eluting stents and drug coated balloons (DCB) to inhibit smooth muscle cell proliferation – a primary contributor to restenosis.⁴⁻⁷

Paclitaxel is a lipophilic drug that is nearly insoluble (0.172 mg/L at pH 7) in water, making it difficult to formulate for intravenous delivery.⁸ Lipophilic vehicles such as polyethoxylated castor oil (Cremophor EL) are used to aid solubility. Unfortunately, Cremophor EL is toxic; it causes vasodilation, labored breathing, lethargy, and hypotension.⁹

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The optimized paclitaxel intravenous formulation consists of 6 mg/mL paclitaxel, polyethoxylated castor oil (Cremophor EL) and anhydrous alcohol.⁸ Although the formulation of paclitaxel has been optimized, solubility, precipitation, and toxicity concerns remain, highlighting the difficulty of working with paclitaxel solutions.

With the recent approval of DCB in the US market, research has moved to optimizing paclitaxel/excipient formulations for arterial paclitaxel delivery.^{10, 11} Paclitaxel excipients are capable of modulating coating durability, paclitaxel solubility/bioavailability, pharmacokinetics, and vascular healing post-treatment.^{12–14} Current excipients include polysorbate and sorbitol, urea, and iopromide.^{15–18} Although these excipients have improved the success of DCBs, long-term drug residency and vascular healing remain subpar. This opens the door for discovery of improved excipients capable of elongating drug residency and improving vascular healing in paclitaxel drug coated balloons.

As keratins are becoming increasingly studied in drug delivery applications, a method is needed to quantify drug release from keratins. Equivalently, methods capable of quantifying paclitaxel from excipients are needed, as excipients of paclitaxel are being evaluated. Keratin is a protein extracted from hair and a widely used biomaterial that represents potential as a novel paclitaxel excipient.^{19–21} Keratin is highly biocompatible, has intrinsic scaffolding capability, supports release of several drugs and factors, and promotes anti-inflammation *in vivo*.^{19, 22–26} Both keratose and kerateine are accumulations of protein/protein fragments and isoforms of various sizes.^{19, 22} The hydrophilic nature and range of molecular weights in keratin preparations complicate mass spectral analysis.

HPLC-MS methods describing the quantification of paclitaxel from biological samples are numerous and effective in dealing with many complex matrices, however a method to quantify paclitaxel from oil-containing samples while maintaining mass spectral selectivity has yet to be described.^{27–29} This paper describes a method for paclitaxel quantification from keratose and polyethoxylated castor oil containing samples. This method can be applied to the quantification of paclitaxel from any aqueous solution containing water-soluble protein and/or oils.

2. Materials and methods

2.1. Chemicals and materials

Paclitaxel Injection, USP was purchased from Sagent Pharmaceuticals (Schaumburg, IL). The internal standard, Docetaxel (purity >99%), was obtained from Tocris Bioscience (Bristol, United Kingdom). Dimethyl sulfoxide (purity 99.7%) and high performance liquid chromatography (HPLC) grade methanol (purity 99.96%), water, acetonitrile (purity 99.9%) and formic acid (purity 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Absolute ethanol 200-proof (purity 99.5%) was purchased from Fisher Scientific (Hampton, NH). Tert-butyl methyl ether was obtained from Acros Organics (New Jersey, US).

2.2. Instrumentation

Samples were evaporated with a CentriVap Vacuum Concentrator (Labconco, Fort Scott, KS). Separation was performed on a Symmetry C18, 100 Å, 3.5 µm, 100 × 2.1 mm column

(Waters Corporation, Milford, MA) using an Accela HPLC Autosampler, Accela 600 LC Pump, and LTQ Velos Orbitrap Mass Spectrometer with Electrospray Ionization (Thermo Scientific, Waltham, MA). The mass spectrometer (MS) was run in positive ion mode.

2.3. Sample Prep

Paclitaxel Injection, USP [6 mg/mL] was combined with phosphate buffered saline (PBS) (GE Healthcare Hyclone, Logan, UT) at a final paclitaxel concentration of 1.43 mg/mL. Lyophilized keratose (KeraNetics, Winston-Salem, NC) was combined with the paclitaxel solution (1.43 mg/mL) as 12, 15, and 20% weight-to-volume mixtures. Using a syringe, keratose-paclitaxel mixtures were injected into 2 mL Eppendorf tubes. Tubes were centrifuged at 1200 rpm for 2 minutes to remove bubbles and then incubated overnight at 37° C to form hydrogels. Each hydrogel (12, 15, and 20%) contained 500 µg of paclitaxel. Upon formation of the hydrogels, 500 µL of 1x PBS were added to each tube. At time points ranging from 1 hour to 45 days, PBS was removed and replaced with fresh PBS. PBS samples were kept at -20°C in a clean, siliconized, low-retention tube until ready for quantification. Prior to quantification, samples were thawed on ice. Samples were diluted in starting mobile phase conditions: acetonitrile-water + formic acid 0.1% (55:45, v/v). Samples were transferred to autosampler vials (SUN-SRi, Rockwood, TN) and 0.5 µg of docetaxel were added as an internal standard to each sample. If paclitaxel quantification was out of the linear calibration range, samples were re-diluted and re-run until they fell within the linear range.

2.4 Calibration and quality control samples

Docetaxel was dissolved in dimethyl sulfoxide at a concentration of 1 µg/µL. Paclitaxel, USP (6 mg/mL) and docetaxel were further diluted in starting mobile phase. Six paclitaxel calibration standards were prepared at concentrations ranging from 0.01 ng/µL to 1.25 ng/µL. Calibration standards and quality control samples were stored at 4°C for no longer than one month. Quality control samples were prepared at a concentration of 0.2 ng/µL paclitaxel. Prior to mass spectrometric analysis, all samples, including calibration standards and quality control samples, received an addition of 0.5 µg docetaxel to 95 µL of sample to serve as the internal standard.

2.5. Extraction procedure

To quantify paclitaxel concentration in each sample, keratose needed to be removed. First, samples were thawed on ice, then 200 µL were pipetted into a clean, siliconized, low-retention micro-centrifuge tube. Keratose was precipitated from each sample by the addition of ethanol. One mL of 200-proof ethanol was added to each tube. Tubes were inverted several times, sonicated (Branson Ultrasonics, Danbury, CT) for one minute and then centrifuged for 3 minutes at 1200 rpm. Keratose collected at the bottom of the tube. The supernatant containing the paclitaxel was transferred to a clean tube and dried in the Vacuum Concentrator at 30°C, 1700 rpm. Sample evaporation took 3–3.5 hours. Dried samples were redissolved in mobile phase. Samples were transferred to autosampler vials and 0.5 µg of docetaxel were added as an internal standard to each sample.

2.5. Selection of extraction solvent

Ethanol and tert-butyl methyl ether were evaluated as extraction solvents. Ethanol extraction was evaluated as a method for protein precipitation, and tert-butyl methyl ether extraction was evaluated as a method of liquid-liquid extraction. Ethanol extraction was performed as described above. For extraction with tert-butyl methyl ether, 1 mL of tert-butyl methyl ether was added to each sample. Tubes were inverted several times, sonicated for 1 minute, and then allowed to rest for 2 minutes. The tert-butyl methyl ether, organic phase was decanted into a new tube, vacuum dried, and re-suspended in mobile phase for HPLC-MS.

2.6. Chromatographic and mass spectrometric conditions

Docetaxel was chosen as the internal standard based on its structural similarity to paclitaxel (Fig 1). The molecular ion ($m/z = 853.9$ for paclitaxel and $m/z = 807.9$ for the internal standard) was used to identify each analyte. The mobile phase consisted of acetonitrile as mobile phase A and 0.1% formic acid in water as mobile phase B. The following gradient was applied: 45% A (0–12 min), from 45 to 100% A (12–15 min), 100% A (15–19 min), from 100 to 45% A (19–24 min), and 45% A (24–38 min). The flow rate for separation was 0.2 mL/min. From minute 13–33 of the separation gradient, flow was diverted to waste to prevent polyethoxylated castor oil from entering the electrospray ionization source of the mass spectrometer. Flow was diverted back to the mass spectrometer at 33 minutes, and the column was re-equilibrated with mobile phase for five minutes before the next injection. Sample injection volume was 5 μL and injection solvent was methanol-water (50:50, v/v) because paclitaxel and docetaxel are soluble in such a solution. Mass spectrometer detection settings are listed in Table 1. All samples were analyzed in duplicate. Data processing was performed on Thermo Xcalibur data system (Thermo Scientific, Waltham, MA). Parameters for peak quantification were standardized for all analyses.

2.7. Method validation

The method was evaluated for specificity, signal carryover, intra- and inter-day precision, and calibration curve. Specificity was evaluated by comparing chromatograms of blank, ethanol-extracted samples with paclitaxel spiked samples. Carryover was assessed by six replicate injections of blank, ethanol-extracted samples following an injection of a paclitaxel spiked sample. Intra-day and inter-day precision were evaluated by six replicate injections and evaluated as percent relative standard deviation (%RSD). The calibration curve was formed by graphing the ratio of the peak area of paclitaxel to peak area of internal standard (docetaxel) versus the concentration of paclitaxel. The linear region of detection was determined by preparing a range of paclitaxel concentrations between 0.001 and 150 ng/ μL .

2.8. Statistical analysis

The data were presented as means and standard deviation. Difference of significance was set at 0.05.

3. Results and discussion

Ethanol and TBME were compared as extraction solvents for samples containing varying concentrations of paclitaxel and keratose. Average extraction efficiency of samples

containing keratose and paclitaxel was $31.9 \pm 2.3\%$ ($n=3$) extracted with TBME and $86.4 \pm 4.5\%$ ($n=3$) for samples extracted with ethanol. This led to the selection of ethanol as the extraction solvent. Extraction efficiency of samples from which keratose was precipitated was $93.0 \pm 3.4\%$ ($n=32$). The extraction efficiency is comparable to other HPLC-MS methods for paclitaxel quantification from serum which range from 73% to 98%.^{30–33} Keratose concentration was evaluated for effect on paclitaxel extraction efficiency. At low keratose concentration ($1 \mu\text{g}/\mu\text{L}$) paclitaxel extraction efficiency was $94.3 \pm 3.4\%$ at $4.75 \text{ ng}/\mu\text{L}$ paclitaxel and $94.1 \pm 2.5\%$ at $14.25 \text{ ng}/\mu\text{L}$ paclitaxel. For high keratose concentration ($5 \mu\text{g}/\mu\text{L}$) extraction efficiency was $91.1 \pm 3.9\%$ at $4.75 \text{ ng}/\mu\text{L}$ paclitaxel and $92.4 \pm 3.1\%$ at $14.25 \text{ ng}/\mu\text{L}$ paclitaxel. Keratose did not significantly affect the paclitaxel measurement ($p=0.3987$).

Based on previous methods,^{28, 34–36} HPLC-MS/MS was initially performed with solutions of docetaxel [$5 \text{ ng}/\mu\text{L}$] and paclitaxel [$0.15 \text{ ng}/\mu\text{L}$] with a mobile phase of acetonitrile-water + formic acid 0.1% (45:55, v/v) with isocratic flow at $200 \mu\text{L}/\text{min}$ through a Waters Symmetry C-18 column. The gradient and flow rate were adjusted to optimize peak resolution and decrease run time. Docetaxel and paclitaxel produced well-established peaks without fronting or tailing, retention times were 7.86 minutes (min) and 9.10 min respectively (Fig 2c, 2d). Other elements that make up the paclitaxel IV solution, including polyethoxylated castor oil, eluted after the paclitaxel peak (Fig 2a). The polyethoxylated castor oil carries over into following blank, methanol injections. To prevent this carryover, acetonitrile was ramped up to 100% following paclitaxel and docetaxel elution and, concomitantly, flow was diverted to waste. Mobile phase was then returned to starting conditions, and flow was diverted back to the MS. The column was re-equilibrated before the next injection. Following this method, blank/methanol injections after spiked paclitaxel injections exhibited no carryover signal.

Specificity was determined by comparing chromatograms of blank, ethanol-extracted samples with paclitaxel spiked samples. No overlapping peaks were observed.

No carryover was detected when a blank/methanol sample was injected following a docetaxel spiked sample. Following a spiked paclitaxel injection, no paclitaxel carried over to the blank, but some of the late eluting additive of paclitaxel were carried over.

Quality control (QC) samples were interspersed every five samples at an intermediate concentration of $0.02 \text{ ng}/\mu\text{L}$. Precision of QC samples was 12 %RSD for paclitaxel and 5.4 %RSD for paclitaxel normalized to the internal standard, docetaxel.

The lowest concentration of paclitaxel that could reliably be identified was $0.01 \text{ ng}/\mu\text{L}$. Standards extracted from keratose-paclitaxel samples gave a linear calibration curve from 0.01 to $1.25 \text{ ng}/\mu\text{L}$: the R^2 value was 0.9992 ± 0.0004 ($n=12$).

Inter-day precision for paclitaxel peak area was 13 %RSD. Inter-day paclitaxel peak area normalized to internal standard peak area was 9.1 %RSD. Intra-day precision for paclitaxel peak area was 7.9 %RSD and intra-day precision for normalized paclitaxel peak area was 4.3 %RSD. These %RSD values are consistent with reported values (between 1.04 and 12.3 %RSD) in determination of paclitaxel concentration from plasma samples.^{30–33}

Paclitaxel release from excipients as evaluated here is relevant to paclitaxel delivery, specifically drug coated balloon development. The method has broader applications yet in the quantification of clinical grade paclitaxel IV injection solution through any means of delivery. Although paclitaxel is an accepted therapeutic in the treatment of restenosis, paclitaxel is more commonly used as a cancer therapeutic. Cancer therapeutics are historically delivered systemically, however systemic delivery leads to off-target effects which are of significant concern, considering the toxicity of paclitaxel and Cremophor EL.⁹ This has led to the exploration of targeted drug delivery of cancer therapeutics to minimize negative off-target effects. As cancer treatment heads towards local drug delivery, methods for quantification of paclitaxel from the tumor microenvironment will be critical in the evaluation of these treatments. Our method matches or improves reported methods on the quantification of paclitaxel from water-soluble matrices while achieving clean-up of residual solvents such as castor oil. These methods are invaluable in the pursuit of localized paclitaxel delivery and quantification.

4. Conclusions

A protocol for removing solutes which are insoluble in organic solvents and quantifying paclitaxel from the complex, paclitaxel IV injection solution (Sagent Pharmaceuticals) using HPLC-MS was developed. The method was validated based on linear range (0.01 to 1.25 ng/ μ L) and intra- and inter-day precision (4.3 and 7.9 % respectively). As complex paclitaxel/excipient combinations become more prevalent in the study of local drug delivery, this method will allow the evaluation of drug elution *in vitro*. The authors have no conflicts of interest.

Acknowledgments

Funding

This study was supported by American Heart Association [#15SDG25880000, #16PRE27350003], National Institute of Health [#1R15HL127596] and internal funding from the University of South Alabama.

The authors would like to thank Shelby Boyd, BS for her help in optimizing mass spectrometer settings.

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Highlights

- Paclitaxel was quantified from keratin containing samples via HPLC-MS/MS.
- Tert-butyl methyl ether and ethanol were evaluated as extraction solvents.
- The method was validated based on linear range and intra- and inter-day precision.
- The developed method is applicable to any aqueous paclitaxel samples.

Figure 1. Chemical structure of (a) paclitaxel and (b) docetaxel

Paclitaxel and docetaxel have similar structures but differ in molecular weight because of the difference between a phenyl and a tert-butyl ether attachment on the carbonyl carbon of the amide. In addition, paclitaxel also has an acetate in place of a hydroxyl sidechain in the vinyl position on the main backbone.

Figure 2. Chromatogram of paclitaxel elution

(a and b) Elution of paclitaxel spiked solution with mobile phase acetonitrile-water + formic acid 0.1% (50:50, v/v) with a flow rate of 400 $\mu\text{L}/\text{min}$. a) Total ion chromatogram, b) Selected ion chromatogram for paclitaxel ($m/z = 854$). (c and d) Analyte peaks with mobile phase acetonitrile-water + formic acid 0.1% (45:55, v/v) at 200 $\mu\text{L}/\text{min}$. c) Selected ion chromatogram for docetaxel ($m/z = 808$), d) Selected ion chromatogram for paclitaxel ($m/z = 854$).

Table 1

General MS settings.

<u>Ion Optics (V)</u>		<u>Ion Detection Settings</u>	
Multiple 00 Offset	−0.04	Dynode (kV)	−0.01
Lens 0	−1.34	Multiplier 1 (V)	0.00
Multipole 0 Offset	−8.19	Multiplier 2 (V)	0.92
Lens 1	−9.53		
Front Lens	−7.26	<u>Vacuum</u>	
Back Lens	−9.52	Ion Gauge (Torr)	1.48×10^{-5}
		Convection Gauge (Torr)	1.49
<u>Heated ESI Source</u>		<u>Main RF</u>	
Spray Voltage (kV)	0.01	Main RF Detected (V)	−0.00
Spray Current (μA)	0.02	RF Detector Temp (°C)	54.40
Source Heater Temp (°C)	39.23	RF Generator Temp (°C)	35.66
Capillary Temp (°C)	300.07		