



NCL Method PHA-2

Ultrafiltration Drug Release Assay Utilizing a Stable Isotope Tracer

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

During early development of a nanomedicine, it is important to evaluate the formulation's stability and drug release in biological matrices. In addition, quantifying the encapsulated and unencapsulated nanomedicine drug fractions is important for the determination of bioequivalence (pharmacokinetic equivalence) of generic nanomedicines (1). Since plasma protein binding for most marketed drugs is in excess of 70% (2) and can change in a concentration-, time-, and even formulation- dependent manner (3), accurate determination of the protein bound fraction is a considerable challenge. This assay utilizes an improved ultrafiltration method for nanomedicine fractionation in plasma, in which a stable isotope tracer is spiked into a nanomedicine containing plasma sample in order to precisely measure the degree of plasma protein binding (4) (Figure 1). Determination of protein binding then allows for accurate calculation of encapsulated and unencapsulated nanomedicine drug fractions, as well as free and protein-bound fractions.

This protocol is written to conduct an in vitro drug release study in human plasma, comparing drug release of a bilayer-loaded docetaxel (DTX) nanoliposome to the commercial DTX formulation, Taxotere®, and solvent solubilized DTX. Comparison data using this method were published previously (4). Additionally, the method can be applied to fractionate nanomedicine containing plasma from an in vivo pharmacokinetic study, such as for a bioequivalence trial. As long as a stable isotope labeled version of the free drug is available, the methods introduced here can be tailored to other nanomedicines. The stable isotope tracer is non-radioactive and generally a deuterated or carbon-13 isotope labeled analog of the normoisotopic drug encapsulated in the nanomedicine formulation. It is important that the isotope tracer is at least 3 amu different from

the normoisotopic drug to ensure accurate mass separation and quantitation by mass spectroscopy.

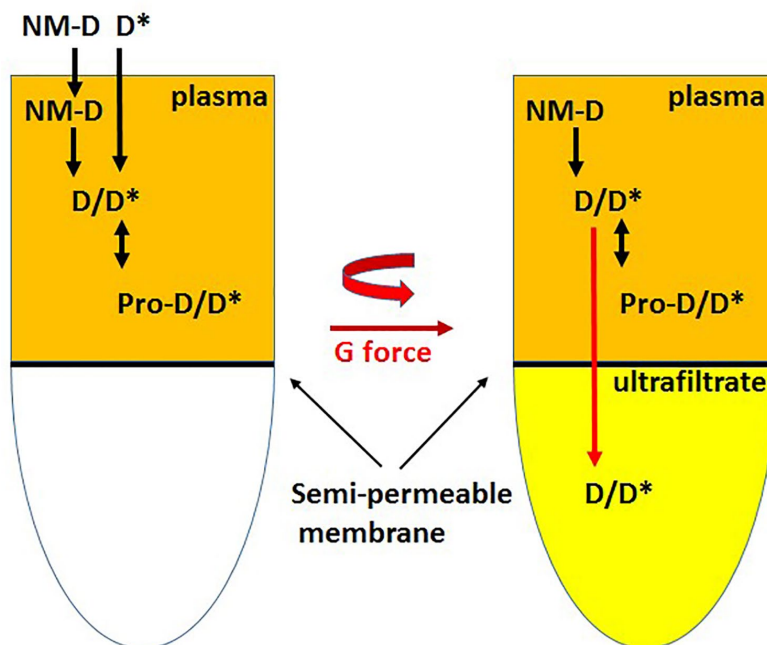


Figure 1. Drug release assay using a stable isotope tracer ultrafiltration method. After the stable isotopically labeled drug (D^*) is spiked into nanomedicine (NM-D) containing plasma, D^* behaves identically to normoisotopic drug (D) with regard to protein binding (Pro-D/ D^*). After protein binding equilibrium is reached, the plasma sample is transferred to an ultrafiltration device and the filtrate is separated by centrifugation. The stable isotope tracer free fraction, represented as the ultrafiltrate fraction, can be used to calculate protein bound, unencapsulated and encapsulated drug fractions, according to equations (i), (ii) and (iii), respectively.

2. Principles

The purpose of this assay is to measure the encapsulated and unencapsulated nanomedicine fractions after incubation in human plasma. In short, a nanomedicine-containing plasma sample is prepared or collected from an in vivo pharmacokinetic study. Then, a stable isotope-labeled drug is spiked into the plasma and allowed to equilibrate with plasma components (**Figure 1**). An aliquot of the sample is taken for analysis of total drug (**Reservoir D**), and the remaining sample is transferred to an ultrafiltration apparatus for collection of the filterable fraction by centrifugation. The filtrate (**Ultrafiltrate D**) is used to measure free/unbound drug. Both the initial aliquot (**Reservoir D**) and the filtrate (**Ultrafiltrate D**) are analyzed by mass spectrometry to determine both the formulation (normoisotopic) and stable isotope tracer drug concentrations. Since the stable, isotopically labeled drug (**D***) and unlabeled, normoisotopic drug (**D**) released from the nanomedicine formulation equilibrate with protein and formulation components to the same degree, the ultrafiltrate fraction of the isotopically labeled drug (**Ultrafiltrate D***) is an accurate measure of the free unbound fraction.

Bound fraction can be calculated from equation (i):

$$(i) \quad \% \text{ Bound } D^* = \frac{([Reservoir D^*] - [Ultrafiltrate D^*]) \times 100}{[Reservoir D^*]}$$

The encapsulated and unencapsulated nanomedicine fractions can then be calculated using equations (ii) and (iii):

$$(ii) \quad [Unencapsulated D] = \frac{[Ultrafiltrate D]}{\left(1 - \frac{(\% \text{ Bound } D^*)}{100}\right)}$$

$$(iii) \quad [Encapsulated D] = [Reservoir D] - [Unencapsulated D]$$

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1 Reagents

- 3.1.1 Human plasma (pooled) collected fresh from 6 human donors in K₂-EDTA tubes
- 3.1.2 1 M HEPES buffer solution
- 3.1.3 Acetonitrile (ACN)
- 3.1.4 Docetaxel-d5 (DTX-d5)
- 3.1.5 Docetaxel-d9 (DTX-d9)
- 3.1.6 20 mg/mL docetaxel (Taxotere®), prepared as directed from manufacturer (Sanofi-Aventis Corporation, Bridgewater, NJ)
- 3.1.7 Docetaxel nanoliposome (bilayer loaded) test nanoparticle
- 3.1.8 Formic acid

3.2 Materials

- 3.2.1 4 mL plastic blood collection tubes with K₂-EDTA
- 3.2.2 10 kDa molecular weight cutoff (MWCO) cellulose membrane ultrafiltration device, 0.5 mL capacity
- 3.2.3 30 kDa MWCO centrifugal filter units, 4 mL capacity

3.3 Equipment

- 3.3.1 Liquid chromatography-mass spectrometry (LC-MS) instrumentation
- 3.3.2 C18 high performance liquid chromatography column, 2.1 mm x 100 mm and matching C18 guard column, 2.1 mm x 10 mm
- 3.3.3 Amber glass screw top HPLC vial with fixed Teflon insert and cap
- 3.3.4 Evaporator and concentrator workstation

4. Experimental Procedure

4.1 Protein Binding Comparison between the Normoisotopic Drug and Isotope Tracer

Certain ultrafiltration devices can have high nonspecific binding to the drug of interest, which may skew results. In this example, the device was determined to have low nonspecific binding to DTX (<20%). Specific binding was determined by incubating 1 µg/mL DTX in protein-free plasma at 37°C for 30 min with agitation in ultrafiltration device, centrifuging, and comparing the filtrate DTX concentration to the reservoir DTX concentration.

The time for the normoisotopic drug to reach equilibrium with plasma proteins must also be determined. If the normoisotopic drug is in equilibrium with both protein and formulation components, then the free fraction should not vary over progressive time points. Free DTX equilibration time was determined by incubating various concentrations of free DTX in plasma for 5, 10, 15 and 30 min and identifying the earliest time at which protein binding stabilized. Protein binding is determined by comparing ultrafiltrate (free/unbound drug) drug concentration to reservoir drug concentration ($\% \text{ bound} = \frac{\text{reservoir} - \text{ultrafiltrate}}{\text{reservoir}} \times 100$). For DTX, the equilibration time was found to be 10 min, which was then used in the stable isotope tracer protein binding comparison study below. The amount of time for free drug to come to equilibrium with the protein bound form is generally from 10-30 min.

Next, the protein binding characteristics of the stable isotope tracer needs to be evaluated to ensure that the stable isotope behaves identically to the normoisotopic drug.

Deuteration, for instance, can lead to changes in the physicochemical properties of drugs that can potentially influence the protein binding characteristics.

- 4.1.1 Collect and pool human blood in K₂-EDTA tubes from 6 donors.
- 4.1.2 Prepare plasma from the pooled blood by centrifugation at 2500xg for 10 min. Add 50 µL of HEPES buffer for every 2 mL of plasma and adjust the pH to 7.4. To prepare protein-free plasma, transfer plasma into a 4 mL centrifugal filter unit with a 30 kDa MWCO, centrifuge for 5000xg for 1 h, and collect filtrate.
- 4.1.3 Solubilize DTX in ACN and spike into 1 mL of prewarmed plasma samples (37 °C) in triplicate to yield final DTX concentrations of 0.5, 1, 2, 5, and 10 µg/mL, keeping organic concentrations <0.5%)
- 4.1.4 Add DTX-d5 solubilized in ACN to the plasma samples to yield a final DTX-d5 concentration of 0.5 µg/mL, keeping organic concentrations <0.05%. The 0.5 µg/mL DTX-d5 spike concentration was determined to be the limit of detection for the unbound stable isotope, with an unbound concentration of approximately 30 ng/mL (~6% unbound).
- 4.1.5 Add 400 µL of plasma samples to prewarmed 10 kDa MWCO centrifuge devices and incubate for 10 min at 37°C with agitation. Spin samples at 6000xg for 10 min.

- 4.1.6 Analyze 50 μL of the ultrafiltrate by LC-MS. Plasma samples (400 μL) incubated in centrifuge devices at 37°C and not spun are also analyzed by LC-MS to determine total drug concentration in the reservoir. There is the potential for drug degradation during the ultrafiltration process. This in-apparatus incubation control accounts for this potential degradation. LC-MS analysis method is described in Section 4.4.
- 4.1.7 Calculate percent bound drug for normoisotopic drug and stable isotope tracer using equation (i).
- 4.1.8 Compare percent bound drug values between the stable isotope tracer and the normoisotopic drug. Ideally values would be within 15% of each other.

4.2 Drug Release Quantitation in Human Plasma

The following protocol is written to compare drug release of DTX loaded liposomes to commercial Taxotere™ and ACN solubilized DTX. The time points chosen in advance were zero, 10, 30 and 60 min. These may be adjusted depending on the drug release kinetics. The concentrations of DTX equivalents chosen were 2, 5 and 10 $\mu\text{g}/\text{mL}$, as they were clinically relevant concentrations based upon its clinical dose and pharmacokinetic profile. The concentrations of the nanomedicine studied should be clinically relevant, based on the actual or expected pharmacokinetic profile.

- 4.2.1 Collect and pool human blood in K₂-EDTA tubes from 6 donors.

- 4.2.2 Prepare plasma from the pooled blood by centrifugation at 2500xg for 10 min. Add 50 μ L HEPES buffer for every 2 mL of plasma and adjust the pH to 7.4.
- 4.2.3 Spike 4 mL of prewarmed plasma samples (37 °C) with DTX liposome, commercial Taxotere, or ACN solubilized DTX in triplicate to yield final DTX concentrations of 2, 5 and 10 μ g/mL in glass vials. Incubate samples for zero, 10, 30 and 60 min at 37°C with agitation.
- 4.2.4 At each time point, spike 400 μ L aliquots of the plasma samples with DTX-d5 to make a final DTX-d5 concentration of 0.5 μ g/mL and vortex. Transfer sample to 10 kDa MWCO centrifuge devices and incubate for 10 min at 37°C with agitation.
- 4.2.5 Centrifuge samples at 6000xg for 10 min and analyze 50 μ L of the ultrafiltrate by LC-MS. Plasma samples (400 μ L) incubated in centrifuge devices at 37°C with agitation and not spun were also analyzed by LC-MS to determine total drug concentration in the reservoir. The LC-MS analysis method is described in Section 4.4.
- 4.2.6 Calculate unencapsulated and encapsulated drug fractions according to equations (ii) and (iii), respectively.

4.3 Control Studies

Studies should incorporate spike recovery controls to insure validity of results. To determine the accuracy of the unencapsulated drug estimation, free normoisotopic drug can be spiked

into plasma with the formulation (*spike recovery study*). A control study to examine the possibility of processing artifacts with regard to encapsulated drug release can be performed by double processing the formulation containing plasma, in which a single formulation containing plasma sample undergoes two successive filtration processes (*double spin study*). Lastly, a control for the organic spike can be performed whereby identical formulation containing plasma samples are compared, in which one sample is spiked with stable isotope and the other is not (*organic stable isotope spike study*). If the the organic spike does not disrupt the formulation, then the calculated % protein binding of the normoisotopic drug in both samples should be identical, ideally within 15% of each other.

- 4.3.1 Collect and pool human blood in K₂.EDTA tubes from 6 donors.
- 4.3.2 Centrifuge the pooled blood at 2500xg for 10 min to prepare plasma. Add 50 µL of HEPES buffer for every 2 mL of plasma and adjust the pH to 7.4.
- 4.3.3 Spike three sets of 4 mL prewarmed plasma samples (37 °C) in glass vials with DTX liposome in triplicate to yield final concentrations of 600 ng/mL. The concentrations of the nanomedicine chosen should be clinically relevant and based on the actual or expected pharmacokinetic profile.
- 4.3.4 Incubate samples for 10 min at 37°C with agitation. The three sets are used for: 1) *double spin study*, 2) *300 ng/mL spike recovery study*, and 3) *organic stable isotope spike study*.

4.3.5 *Double Spin Control Study*

1. Spike DTX-d5 into 400 μL aliquots of plasma from set one to make a final DTX concentration of 0.5 $\mu\text{g}/\text{mL}$ and vortex.
2. Transfer the spiked samples to a 10 kDa MWCO centrifuge device and incubate for 10 min at 37°C with agitation.
3. Centrifuge the samples at 6000xg for 10 min.
4. Analyze 50 μL of the ultrafiltrate by LC-MS (see LC-MS method in Section 4.4).
5. Transfer the reservoir with samples to new centrifuge tubes, spin samples again at 6000xg for 10 min, and collect and analyze a second 50 μL sample of the ultrafiltrate.
6. Plasma samples (400 μL) incubated in centrifuge devices at 37°C with agitation and not spun are also analyzed by LC-MS to determine total drug concentration in the reservoir. This in-apparatus incubation control accounts for the potential for drug degradation during ultrafiltration.
7. Calculate the unencapsulated DTX concentrations according to equation (ii).

4.3.6 Spike Recovery Control Study

1. Spike 300 ng/mL of free DTX and 0.5 $\mu\text{g}/\text{mL}$ of DTX-d5 into 400 μL plasma aliquots from set two and vortex.

2. Transfer samples to 10 kDa MWCO centrifuge devices and incubate for 10 min at 37°C with agitation.
3. Centrifuge samples at 6000xg for 10 min. analyze 50 µL of the ultrafiltrate by LC-MS (see LC-MS method in Section 4.4).
4. Plasma samples (400 µL) incubated in centrifuge devices at 37°C and not spun are also analyzed by LC-MS to determine total drug concentration in the reservoir. This control accounts for the potential for drug degradation during ultrafiltration.
5. Determine unencapsulated DTX concentrations according to equation (ii).
6. To determine spike recovery, the mean of the calculated unencapsulated DTX concentrations for the first spin of the double spin study (step 4.3.5) is subtracted from the mean unencapsulated DTX concentrations of the spiked sample concentrations:

Spike recovery = (unencapsulated DTX concentration of spike sample – unencapsulated DTX concentration of 1st spin)

Ideally, the calculated difference of the spike recovery samples would be within 15% of the theoretical value.

4.3.7 Organic Stable Isotope Spike Study

1. Take 400 µL of plasma aliquots from set three and do **not** spike with ACN solubilized DTX-d5. Vortex the sample and transfer to 10 kDa

MWCO centrifuge devices. Incubate samples for 10 min at 37°C with agitation.

2. Centrifuge samples at 6000xg for 10 min. Analyze 50 µL of the ultrafiltrate by LC-MS (see LC-MS method in Section 4.4).
3. Plasma samples (400 µL) incubated in centrifuge devices at 37°C with agitation and not spun were also analyzed by LC-MS to determine total drug concentration in the reservoir.
4. Determine percent protein binding of the normoisotopic drug, calculated as:

$$\% \text{ protein binding} = \frac{([\text{Total DTX in reservoir}] - [\text{DTX in ultrafiltrate}])}{[\text{Total DTX in reservoir}]} \times 100,$$

for set one (Section 4.3.5) and set three (Section 4.3.7) in order to determine the effect of organic spike on formulation stability. Ideally, the percent protein binding values between the two sets would be within 15% of each other.

4.4. LC-MS Set-Up and Analysis

- 4.4.1 Set HPLC conditions at: 5 µL injection volume, water-ACN gradient (30% ACN/0.1% formic acid from 0-1.5 min, linear increase to 80% ACN/0.1% formic acid from 1.5-4.5 min, hold at 80% ACN/0.1% formic acid from 4.5-8.5 min, and linear decrease to 30% ACN/0.1% formic acid from 8.5-10.5 min), flow rate of 0.35 mL/min, and column temperature of 32°C. The column regeneration time between injections is 6.5 min.

4.4.2 Use an MS instrument with an electrospray ionization source in positive ion mode. Set detector voltage at 0.2 kV and the desolvation line (DL) and heat block temperature at 200°C. Use high pressure liquid nitrogen as the drying gas at a rate of 1.5 L/min. DTX, DTX-d5 and DTX-d9 elution times were all 8.9 min, and m/z ions monitored by selected ion monitoring (SIM) were 808, 813 and 817 respectively.

4.4.3 Measure the peak area ratio of the analyte to internal standard, DTX-d9, and use it to interpolate DTX concentrations of unknowns from a linear fit of calibration curves. The calibration curve range can vary depending on the assay analyte concentrations using calibration standards prepared in appropriate assay matrix. For each calibration run, include quality control samples from the low, mid and high points of the calibration curve prepared in appropriate assay matrix. For specifics as to how the calibration and quality control samples are to be prepared, please refer to sub-sections 4 and 5 below.

4.4.4 Calibration and Quality Control Standards Preparation for LC-MS
Analysis

An additional requirement of the method is a second stable isotope, DTX-d9, that is used as an internal standard to allow for accurate quantitation of the normoisotopic drug and stable isotope tracer by mass spectrometry. Again, this stable isotope should be at least 3 amu different from both the normoisotopic drug and the stable isotope tracer to allow for accurate mass separation and quantitation.

1. Make stock solutions of DTX, DTX-d5 and DTX-d9 for calibration and quality control standards by solubilizing in ACN.
2. Prepare DTX and DTX-d5 calibration standards in human plasma and protein-free plasma at concentrations ranging from 25 to 25,000 ng/mL. Spike DTX-d9 as an internal standard at a concentration of 250 ng/mL.
3. Prepare DTX and DTX-d5 in human plasma and protein-free plasma in duplicate with concentrations of 125, 1,000 and 10,000 ng/mL DTX as low, medium and high quality control standards, respectively. The average of the duplicate concentrations would ideally be within 15% of theoretical, with %CV <15%.

4.4.5 *Sample Preparation with Controls for LC-MS Analysis*

1. Add 50 µL of sample or calibration standard spiked with 250 ng/mL of DTX-d9 internal standard to a 2 mL eppendorf tube, followed by addition of 200 µL of ice cold ACN with 0.1% formic acid. Vortex.
2. Place the sample in -80°C for 10 minutes and then thaw at room temperature.
3. Centrifuge the thawed sample at 14,000xg for 20 min at 4°C to pellet precipitated protein.
4. Transfer the supernatant to a glass tube and dry under nitrogen gas in a concentrator workstation at 48°C.
5. Resuspend the dried residue in 150 µL 30% ACN with 0.1% formic acid.
6. Transfer the extracted sample to a 0.5 mL eppendorf tube and centrifuge at 14,000xg for 5 minutes at room temperature.

7. Transfer the supernatant to a 1.5 mL amber glass screw top HPLC vial with fixed Teflon insert and cap and place in an HPLC autosampler vial rack.
8. Run plasma sample blank (plasma only), internal standard spiked plasma blank (plasma spiked internal standard) and quality control samples with each calibration curve. Follow the LC-MS method in Section 4.4.

5. References

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6. Abbreviations

ACN	Acetonitrile
D*	Isotopically labeled drug
D	Unlabeled, normoisotopic drug
DL	Desolvation line
DTX	Docetaxel
HPLC	High-performance liquid chromatography
K ₂ -EDTA	Di-potassium ethylenediaminetetraacetic acid
LC-MS	Liquid chromatography-mass spectrometry
MWCO	molecular weight cutoff
NCL	Nanotechnology Characterization Laboratory
SIM	Selected Ion Monitoring