NCL Method PHA-1

Radioactive Blood Partitioning Assay

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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

Characterization of drug release under physiological conditions is very important for prediction of drug release in vivo, and can be used for formulation optimization. This assay uses a blood partitioning technique to measure the release of free drug from a nanoparticle-encapsulated drug complex. This assay is based on the principle that the free drug will partition between the plasma and red blood cell (RBC) fractions, whereas the hydrophilic nanoparticle platform is generally retained in the plasma fraction.

The protocol is written for analysis of a dual radioactively labeled drug-nanoparticle platform complex (i.e. ^{14}C-labeled drug and ^{3}H-labeled nanoparticle platform). The protocol can also be adapted to use non-radioactive materials, provided that suitable analytical methods can be developed to measure both the drug and platform components independently in blood and plasma matrix (i.e. ICP-MS for the nanoparticle platform component and HPLC for the drug component). The same principles outlined in the study design below could be used for these non-radioactive analytical methods.

2. Principles

The purpose of this study is to determine the extent of RBC partitioning of a dual radiolabel ^{14}C-drug:^{3}H-platform nanoparticle complex, following incubation of whole blood with the dual labeled nanoparticle complex. By measuring both ^{14}C-drug:^{3}H-platform signal in the whole blood and plasma fractions, the extent of component partitioning into plasma and RBC fractions of whole blood can be calculated by the equation:

$$F_p\% = (C_p / C_b) \times (1 - H_c) \times 100 \qquad \textbf{Equation 1}$$

where C_p is the concentration in plasma, C_b is the concentration in whole blood, and H_c is the Hematocrit. F_p%, the fraction in plasma, is an estimate of the percentage in the plasma fraction. Whereas, (100–F_p%) would be an estimate of the percentage in the RBC fraction.

Another important parameter is the ratio of the $^{14}C/^{3}H$ signals as a measure of platform integrity. If the nanoparticle complex is stable, then the $^{14}C/^{3}H$ signal ratio should be constant in all fractions measured, and F_p % should be the same for drug and platform. If the drug component partitions independent of platform, then this ratio in the blood fractions will change and F_p % will be disparate for drug and platform.

Hydrophilic platforms (e.g. PEGylated materials) are generally retained in the plasma fraction. As this assay does not involve "sink" conditions, partition of the radiolabeled drug:platform complex should be compared to partitioning of free radiolabeled drug in order to determine at what F_p% equilibrium between the red blood cells and plasma occurs (this represents the maximum drug release attainable in the assay). Knowledge of the percentage of free drug in the initial nanoformulation is important in evaluating the actual drug release.

3. Reagents, Materials, Cell Lines, 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 15 mL female heparinized Sprague Dawley (SD) rat (or human) whole blood (rat whole blood, Innovative Research, SKU: IR1-150N Li-Heparin; human whole blood, Innovative Research, SKU: IPLA-WB1 Li-Heparin)

3.1.2 ^{14}C-drug/^{3}H-platform dual labeled nanoparticle complex (Theoretical per mL activity of the reconstituted nanoparticle complex should be ~ **7.5 μCi ^{14}C-drug/ 15 μCi ^{3}H-platform/mL**)

3.1.3 ^{14}C-free drug (Theoretical per mL activity of the reconstituted nanoparticle complex should be ~ **7.5 μCi ^{14}C-drug/mL**)

3.1.4 BTS-450 tissue solubilizer (500 mL, Beckman Coulter, P/N 580691)

3.1.5 Ready Organic Scintillation Cocktail (3.75L, Beckman Coulter, P/N 586600)

3.2 Materials

3.2.1 1.5 mL Li-heparin coated-Eppendorf tubes (Fischer Scientific, 05-407-13C)

3.3 Equipment

3.3.1 Barnstead Thermolyte Labquake Shaker

3.3.2 37˚C oven

3.3.3 VWR Galaxy mini centrifuge

3.3.4 Beckman Coulter LS6500 scintillation counter

4. Experimental Procedure

CAUTION: This procedure uses radioactivity. Radioactivity should only be handled by properly trained personnel, using all appropriate safety measures. Disposal of radioactive waste should be done in accordance with your local regulations.

4.1 Reconstitute the dual radiolabeled nanoparticle complex and free radiolabeled drug to 7.5 µCi ^{14}C-drug/ 15 µCi ^{3}H-platform/mL in an appropriate aqueous diluent. Immediately take a small aliquot for DLS size measurement (or other stability indicating assay), and measurement of ^{14}C/^{3}H signal to estimate specific activity and compare to theoretical.

4.2 To determine actual specific activity, add 1 mL of BTS-450 to 10 µL reconstituted nanoparticle complex or free radiolabeled drug, followed by addition of 200 µL of a 30% (w/w) H_2O_2 solution and 15 mL of Ready Organic Scintillation Cocktail. Radioactivity is measured by scintillation counting using dual ^{14}C:^{3}H label mode (Beckman Coulter LS6500).

4.3 Prepare time zero samples, by spiking 100 µL reconstituted nanoparticle complex or free radiolabeled drug into 400 µL of 37˚C whole blood in triplicate, vortex, and immediately collect 10 µL of whole blood (*This is the C_b value at time zero*). Immediately centrifuge the remaining whole blood sample at 4˚C to prepare plasma (VWR Galaxy mini, 2000x*g*, 5 min), and collect 10 µL of resulting plasma for radioactivity measurement (*This is the C_p value at time zero*).

4.4 Prepare the 10 and 60 min time points (or any additional time points desired) by spiking 100 µL of the reconstituted nanoparticle complex or free radiolabeled

drug into 400 µL of whole blood in triplicate, and vortex. Incubate the samples at 37°C on a rotary shaker (set at 8 rpm) for the desired time.

4.5 Following incubation, a 10 µL sample of the radiolabel:blood mixture is removed for radioactivity measurement (*These are the C_b values at each timepoint*). Plasma is separated from the remaining radiolabel:blood mixture by centrifugation (VWR Galaxy mini, 2000x*g*, 5 min), and 10 µL of the resulting plasma is used for radioactivity measurement (*These are the C_p values at each timepoint*).

4.6 Plasma and blood samples are prepared by adding 1 mL of BTS-450 to the 10 µL sample, followed by addition of 200 µL of a 30% (w/w) H_2O_2 solution and 15 mL of Ready Organic Scintillation Cocktail. Radioactivity is measured by scintillation counting using dual ^{14}C:^{3}H label mode (Beckman Coulter LS6500).

5. Calculations

5.1 The fraction of drug and platform in plasma (Fp) is calculated according to Equation 1 [1]. The estimated hematocrit value for female SD rat is (H_c) is 0.44, according to the literature [2]. Ideally, the actual hematocrit of the blood sample should be determined (especially in the case that the drug complex causes hemolysis!). The mean values and standard deviations of the triplicate measurements should be reported.

6. References

1. Petterino C, Argentino-Storino A. (2006) Clinical chemistry and haematology historical data in control Sprague-Dawley rats from pre-clinical toxicity studies. *Exp Toxicol Pathol.* **57(3)**:213-9.

2. Weiss HM, Fresneau M, Camenisch GP, Kretz O and Gross G (2006) In vitro blood distribution and plasma protein binding of the iron chelator deferasirox (ICL670) and its iron complex Fe-[ICL670]2 for rat, marmoset, rabbit, mouse, dog, and human. *Drug Metab Dispos* **34:**971-975.

7. Abbreviations

C_b	concentration in whole blood
C_p	concentration in plasma
DLS	dynamic light scattering
F_p%	fraction in plasma
GTA	general toxicity assay
H_c	Hematocrit
NCL	Nanotechnology Characterization Laboratory
RBC	red blood cell
SD	Sprague Dawley