Evaluating the Impact of Freezing Biologic Fluids on Drug Distribution and Its Impact on Frozen Aliquotting for Quantitative Drug Analysis

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Introduction:

The application of the CXT 750 Automated Frozen Sample Aliquotter to the field of regulated drug analysis has potential for simplifying and streamlining the development, validation and execution of bioanalytical assays. As the cold chain of the parent biospecimen is fully maintained during aliquotting procedures, pre-analytical variability associated with freeze-thaw and extreme temperature differentials is significantly decreased over the lifespan and utilization of the sample. Moreover, the ability to generate quantitative frozen aliquots of a specified volume enables streamlining of the sample preparation for bioanalysis, reducing analytical variability as a result of thawing procedures for compounds where bench stability may be a concern. Ultimately, both advantages of frozen aliquotting may result in faster assay validation timelines, more robust clinical execution and increased pass rates for ISR. Although the advantages above have been demonstrated in previous experiments, the question of how a target analyte distribute within a biological matrix during typical sample freezing procedures and the impact that may have on analytical outcomes when utilizing frozen aliquotting as part of an analytical workflow remains unclear.

Overview:

The advantages of frozen aliquotting for stabilizing labile drug compounds ex vivo for quantitative drug analysis has been previously demonstrated by GSK and CryoXtract. How a drug compound is distributed within the frozen biological matrix as a result of the freezing methodology, biological matrix utilized and the protein binding characteristics of the drug compound may be crucial for the successful integration of frozen aliquotting into any given bioanalytical assay. This study investigates the question of sample homogeneity for the drug compounds atenolol (6-16% protein bound) and (R) - warfarin (99% protein bound) in both frozen serum and EDTA plasma prepared using three different freezing protocols.

Human male serum and K2 EDTA plasma was spiked with either atenolol (1000ng/mL) or (R) - warfarin (100ng/mL) and then aliquotted into flat bottom cryogenic vials and frozen three different ways; SNAP frozen in LN2 vapor, -80°C or -20°C frozen. Samples were cored with the CXT 750 to generate cores at specificed locations and depths to evaluate potential lateral (center versus perimeter core) and vertical (50µL, 100µL and 150µL) gradients. Drug concentrations were determined against a 1-point calibration standard and by comparing analyte peak area ratios to an internal standard by LC-MS/MS analysis for each core and compared to equivalently prepared control samples that were not processed by frozen aliquotting.

Homogeneity: Inter sample CVs ranged between 2.6 - 12.5% for cores and 0.5 - 10.7% for the liquid aliquotting control indicating good performance for a non-GLP assay. There is no strong evidence of a lateral gradient being formed. There is evidence of vertical gradient formation which is associated with an increase in freezing temperature. The average percent difference of top and full cores can be used as a coarse measure of the degree of sedimentation due to freezing conditions.

Control Study: Investigated analyte sedimentation in the sample matrix as a function of ambient bench time between sample mixing and SNAP freezing. The average percent difference in concentration comparing T0 to T60 is reported in Figures 4A - D is 7.6, 0.2, 7.9 and 4.4% respectively. Samples exposed to bench times of 0 - 60 minutes prior to freezing were not observed to have vertical gradients for either atenolol or warfarin in plasma and serum. This suggests that sedimentation is primarily driven by the freeze rate and may be a result of decreased solubility as sample temperature decreases.

Next Steps: Despite the presence of vertical gradients, the reproducibility of drug concentrations from cores suggest that frozen aliquotting can be integrated into the workflow of a validated assay. Two-thirds of cores tested fell within +/- 15% of the average concentration observed in ten of the twelve experiments. The ability to define freezing protocols and target fill volumes during sample collection would allow for a greater degree of assay optimization.

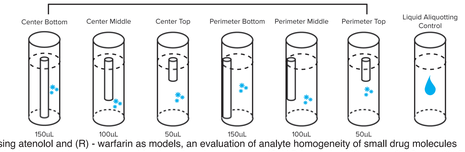
Methodology:

In the pilot study, as described in the abstract, the concentrations of atenolol and (R) - warfarin were determined to be outside the optimal range for peak resolution by LC-MS/MS. Consequently, a lower concentration of atenolol (1000ng/mL) and (R) - warfarin (100ng/mL) was elected for the full study. In addition, the internal standard propranolol used in the pilot study was exchanged for deuterated atenolol and (R) - warfarin for the full study.

Normal, fresh, pooled male human serum and EDTA plasma was sourced from BioreclamationIVT and shipped overnight at 4°C. Upon receipt the serum and plasma was stored at 4°C until aliquotted into flat bottom cryovials at a fill volume of 1.8mL. The plasma and serum was split into two aliquots which were spiked with either atenolol (1000ng/mL) or (R) - warfarin (100ng/mL). The samples were vortex mixed then SNAP frozen in LN2 vapor, -80°C or -20°C frozen. After twenty four hours all samples were stored in the -80°C freezer until processing.

Figure 2 illustrates the experimental design executed to assess sample homogeneity by evaluating the samples for lateral (center versus perimeter core) and vertical (50µL, 100µL and 150µL) gradients utilizing three freezing methodologies utilizing CryoXtract's CXT 750 Frozen Sample Aliquotter (Figure 1) and by employing the targeted volume aliquotting feature. Additionally, a frozen control was placed in the CXT 750 but not cored. This control served as the liquid aliquotting control when samples are assayed by LC-MS/MS.

Figure 2: Sample Aliquotting Methodologies & Comparisons



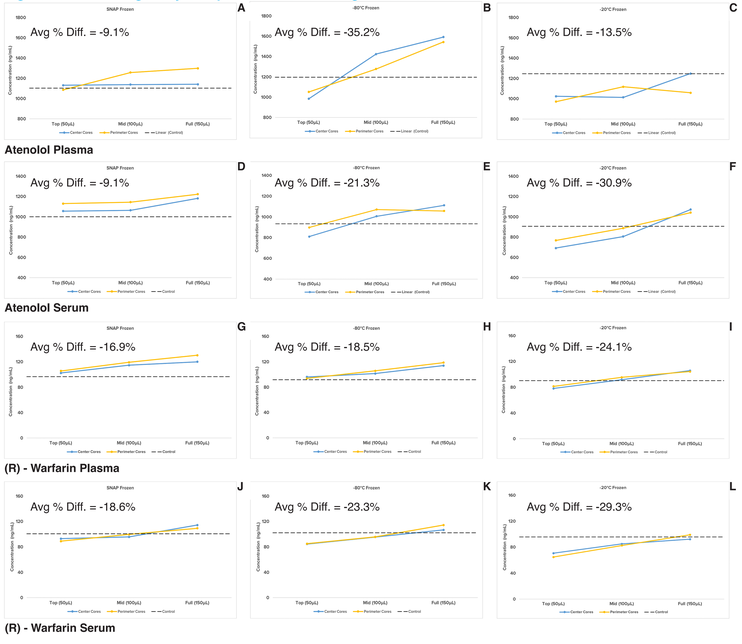
Using atenolol and (R) - warfarin as models, an evaluation of analyte homogeneity of small drug molecules in the frozen matrix was assessed for three freezing conditions (SNAP frozen in LN2 vapor, -80°C and -20°C frozen), two lateral coring positions (center vs. perimeter) and three vertical coring depths (50µL, 100µL and full cores of approximately 150µL) from 1.8mL filled flat bottom cryovials. Each of the test conditions was compared to a liquid aliquotting control, which was treated the same as the test samples but never cored.

After processing on the CXT 750, all cores and controls were stored in the -80°C freezer until delivered to a CRO for processing on dry ice. In preparation for LC-MS/MS analysis, the plasma and serum samples were subjected to a protein precipitation step in which three volumes of methanol containing an analytical internal standard, deuterated atenolol or warfarin, was utilized. Atenolol samples only were diluted ten fold with blank plasma or serum prior to processing to avoid peak saturation in both the full and control study. (R) - warfarin was diluted five fold in the control experiment only. The samples were centrifuged to pelletize precipitated protein and the supernatant was analyzed by LC-MS/MS. All samples were compared to a single point calibration standard (in corresponding plasma or serum). After separation on a C18 reverse phase HPLC column using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using ESI ionization in MRM mode. Data was generated in a non-GLP environment utilizing a non-validated assay.

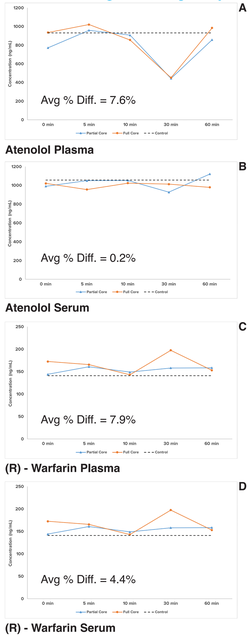
A control experiment was performed to investigate whether bench time prior to SNAP freezing affects vertical gradient formation of small drug molecule spiked plasma or serum. To test this hypothesis, plasma and serum were spiked with either atenolol (1000ng/mL) or (R) - warfarin (100ng/mL) and aliquotted into 1.8mL flat bottom cryovials. Samples were mixed equivalently by inversion then subjected to different ambient bench times (0, 5, 10, 30 and 60min) before SNAP freezing in vapor phase LN2. After SNAP freezing samples were stored overnight at -80°C. Two intravial perimeter cores at two vertical coring depths (50µL and 150µL) were extracted utilizing CryoXtract's CXT 750 Frozen Sample Aliquotter and by employing the targeted volume aliquotting feature. Additionally, a frozen control was placed in the CXT 750 but not cored. This control served as the liquid aliquotting control when samples are assayed by LC-MS/MS. After processing on the CXT 750, all cores and controls were stored in the -80°C freezer until delivered to a CRO for processing. The samples were analyzed using the same methodology described above for the full study.

Results:

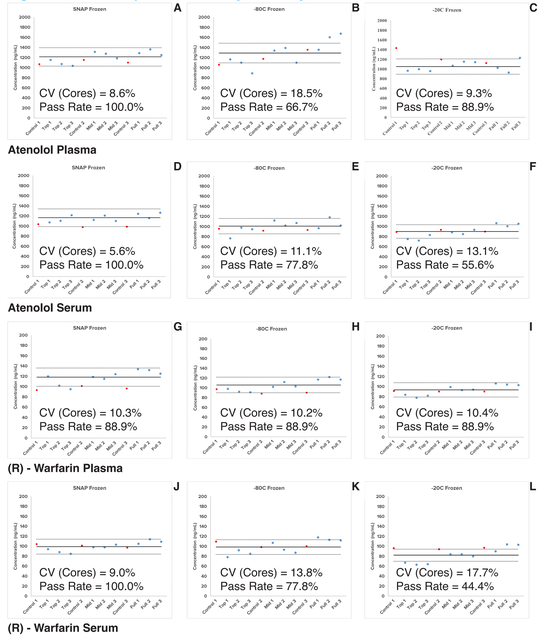
Figures 3A-L: Homegeneity Comparison of Small Drug Molecules in a Frozen Matrix: SNAP vs -80°C vs -20°C Frozen



Figures 4A-D: Bench Time Effects Prior to Freezing on Homogeneity



Figures 5A-L: Extrapolated Bioanalysis Assay Performance



Homogeneity (Figures 3A - L)

- Inter sample CVs range between 2.6 - 12.5% for all cores (perimeter, center, top, middle and full) and 0.5 - 10.7% for the liquid aliquotting control indicating acceptable performance for a non-GLP assay.
- There is no strong evidence of a lateral gradient being formed.
- There is evidence of vertical gradient formation which is associated with an increase in freezing temperature.
- The average percent difference of top and full cores can be used as a coarse measure of the degree of sedimentation due to freezing conditions. The percent difference calculation applies the average value of the perimeter and center cores for the top and full cores respectively.

Control Study (Figures 4A - D)

- Investigated analyte sedimentation in the sample matrix as a function of ambient bench time between sample mixing and SNAP freezing.
- The average percent difference in concentration comparing T0 to T60 is reported in Figures 4A - D is 7.6, 0.2, 7.9 and 4.4% respectively.
- No sedimentation effects were observed due to bench time after mixing and prior to SNAP freezing.

Figure 1: CryoXtract's CXT 750 Frozen Sample Aliquotter

Bioanalysis Potential (Figure 5A - L)

- The data from the homogeneity experiments can be used to illustrate how a bioanalytical method may be developed and validated using frozen aliquotting technology even when a sample type and analyte show tendencies toward vertical gradients.
- The average concentration of the mid core depth (100µL) is used as a reference concentration (solid black line) for each condition and the dashed lines represent the +/- 15% pass/fail threshold based on the reference concentration.
- The blue dots represent each individual perimeter core concentration (top, middle and bottom) obtainied and the concentration from liquid controls are indicated as red dots.
- The coeffecient of variability of concentrations derived from all perimenter cores falls below 15% for all conditions except two, Figures 5F &5L.
- Additionally, the individual cores were also observed to fall within 15% of the reference concentration for 10 out of 12 experiments.

Conclusions:

- Overall, experimental variability as judged by the reproducibility of control samples per experimental condition was low with CVs ranging from 0.5 - 4.7%. The exception was the -80°C and -20°C conditions for atenolol spiked plasma which had CVs of 10.1% and 10.7%, respectively. The variance may be confounding the homogeneity assessment for atenolol plasma at -80°C and -20°C.
- In general, no strong evidence for lateral gradients were observed in any of the test conditions.
- Vertical gradients were observed for all conditions. The degree of analyte sedimentation seems to increase with slower freeze rates.
- Samples exposed to bench times of 0 - 60 minutes prior to freezing were not observed to have vertical gradients for either atenolol or warfarin in plasma and serum. This suggests that sedimentation is primarily driven by the freeze rate and may be a result of decreased solubility as sample temperature decreases.
- Despite the presence of vertical gradients, the reproducibility of drug concentrations from cores suggest that frozen aliquotting can be integrated into the workflow of a validated assay. CVs < 15% were observed for cores in 10 out of 12 experiments.
- Two-thirds of cores tested fell within +/- 15% of the average concentration observed in 10 of the 12 experiments.
- The ability to define freezing protocols and target fill volumes during sample collection would allow for a greater degree of assay optimization.