# *Ex vivo* Stabilization of Small Molecule Compounds and Peptides in EDTA Plasma for LC-MS/MS Analysis Using Frozen Aliquotting

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**Drug development and pharmaceutical research relies heavily on animal and human biospecimens to generate data needed to bring new drugs to market.** However, the sensitivity of some compounds in response to common storage and handling practices can convolute analytical data and obscure scientific outcomes. Development timelines for bioanalytical assays can be extended by weeks, or even months, as a result of efforts to identify stabilization protocols for the target compounds. The CryoXtract automated CXT 750 Frozen Sample Aliquotter can enable an uncompromised cold chain workflow for bioanalysis of thermally labile compounds in biological matrices such as plasma. This study, a collaborative effort between GlaxoSmithKline (GSK) and CryoXtract, demonstrates the improvements frozen aliquotting can provide for the stabilization of small molecule compounds and peptides in a biological matrix intended for bioanalysis.

## Experimental Overview and Results

Small molecule compounds and peptides were spiked into human and rat EDTA plasma. The recovery of each compound was determined by LC-MS/MS initially in the fresh mixture and over four successive rounds of freeze-thaw aliquotting and frozen aliquotting, the latter being performed on the CXT 750.

**Figure 1: Comparison of Analytical Recovery for Compounds Aliquotted on the CXT 750 vs. Liquid Aliquotting**

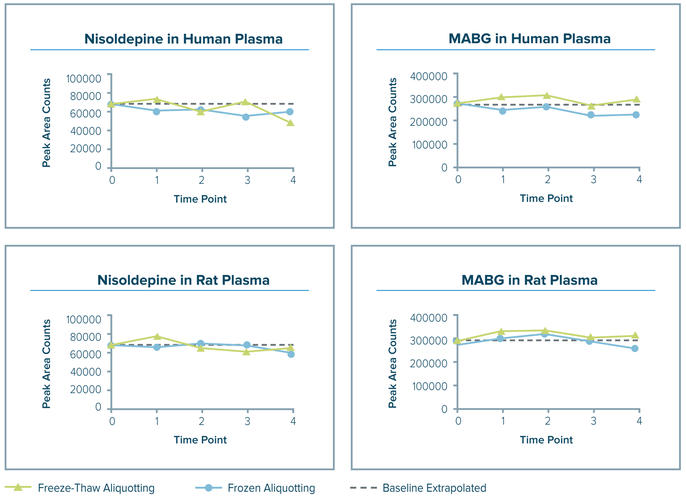


**Table 1: Summary of LC-MS/MS Recovery CVs for $T_0$-$T_4$**

| | HUMAN PLASMA | | RAT PLASMA | |
|---|---|---|---|---|
| | Freeze-thaw | CXT 750 | Freeze-thaw | CXT 750 |
| Nisoldepine | 22.8 % | 8.4% | 11.3% | 8.1% |
| MABG | 8.6% | 7.6% | 6.1% | 9.6% |
| Angiotensin I | 23.9% | 14.6% | 37.2% | 11.5% |
| Caffeic Acid | 93.9% | 11.4% | 98.3% | 13.1% |
| Cisatracurium | 202.1% | 40.6% | 221.5% | 54.1% |

**Precision:** For both nisoldepine and MABG, peak area counts were fairly stable across all time points in rat and human samples. CVs ranged from 6.1 – 22.8% for freeze-thaw aliquots. CVs were below 10% (7.6 – 9.6%) for frozen aliquots produced on the CXT 750 (see Figure 1 and Table1). Frozen aliquotting was observed to be a quantitative method for aliquotting small molecule compounds in frozen EDTA plasma.

**Figure 2: Comparison of Stability for Compounds Aliquotted on the CXT 750 vs. Liquid Aliquotting**

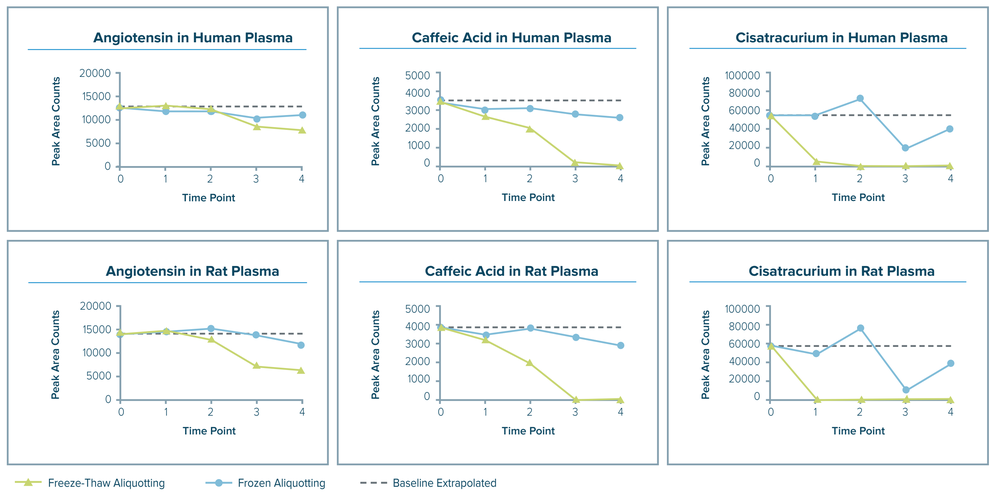


**Table 2: Summary of Percent Recovery at $T_4$**

| | HUMAN PLASMA | | RAT PLASMA | |
|---|---|---|---|---|
| | Freeze-thaw | CXT 750 | Freeze-thaw | CXT 750 |
| Nisoldepine | 71.5 % | 86.8% | 96.6% | 85.0% |
| MABG | 109.8% | 85.8% | 107.7% | 87.5% |
| Angiotensin I | 61.5% | 86.3% | 43.1% | 82.3% |
| Caffeic Acid | 0.3% | 74.9% | 0.0% | 74.6% |
| Cisatracurium | 0.0% | 75.2% | 0.0% | 67.7% |

**Stability:** Peak area counts show a marked decline across time points zero through four in freeze-thaw aliquots (see Figure 2 and Table 2). In comparison, frozen aliquots of human and rat plasma samples produced on the CXT 750 showed significant improvements in stability. Caffeic acid was not recoverable by LC-MS/MS after two to three freeze-thaw cycles, whereas 75% of compound remained recoverable after four frozen aliquotting rounds. Cisatracurium showed little to no detectable amounts by LC-MS/MS after a single freeze-thaw cycle. Although variable, cisatracurium was detectable in all time points when samples were frozen aliquotted.

## Conclusions

Frozen aliquotting has been demonstrated as a valuable tool for compound stabilization and bioanalysis. When analyzing compounds collected in EDTA plasma, the analytical precision associated with frozen aliquotting and the CXT 750 was comparable to liquid aliquotting, and therefore sufficient for quantitative analytical work-flows. Moreover, because the stabilization of highly labile compounds such as caffeic acid and cisatracurium was achieved simply through effective cold chain management via the CXT 750, frozen aliquotting may hold great potential for simplifying sample handling and stabilization protocols in both research and clinical environments, saving valuable time and hastening scientific outcomes.

## Protocol

### Sample Preparation and Treatments

1. The following compounds were spiked into EDTA plasma (human and rat) to the final concentrations as listed in Table 3:

**Table 3: Target Compound Concentrations in Plasma**

| Test Compound | Concentration | Description |
|---|---|---|
| Nisoldepine | 100,000 ng/ml | Small molecule drug susceptible to photochemical degradation[1] |
| MABG | 10,000 ng/ml | Mycophenolic acid beta glucoronide; Antibiotic metabolite susceptible to degradation at physiological pH[1] |
| Angiotensin I | 100 µg/ml | Peptide hormone susceptible to enzymatic degradation |
| Caffeic Acid | 10,000 ng/ml | Naturally derived organic compound susceptible to esterase-induced degradation[1] |
| Cisatracurium | 1000 ng/ml | Small molecule drug susceptible to esterase mediated hydrolysis[1] |

2. Frozen samples were prepared as 1.8ml aliquots in 2.0ml cryogenic vials and frozen at -80°C.
3. Each aliquotting condition (freeze-thaw and frozen aliquotted) consisted of three frozen vials each of spiked rat EDTA plasma and spiked human EDTA plasma.
4. For time points 1-4, samples were removed from the freezer and thawed at room temperature for 1-4 hours (freeze-thaw aliquotting) or placed in the CXT 750 and maintained at -80°C during the frozen aliquotting procedure.
5. For each round of frozen aliquotting, a single core (frozen aliquot), 100µL in volume, was generated per sample on the CXT 750, with a total of four cores removed from each frozen aliquotted sample over the course of the study.

CXT 750 FROZEN SAMPLE ALIQUOTTER

6. The samples were prepped for LC-MS/MS analysis by performing a protein precipitation step according to the following protocol:
   a. 250µl of 1:1 acetonitrile and methanol added to each 100µl aliquot of plasma
      i. 100µl frozen aliquots were added to the protein precipitation step directly and allowed to thaw in the acetonitrile/methanol mixture. 100µl aliquots from the freeze-thaw condition were added in liquid form.
   b. The samples were vortexed for 5 minutes at 1800rpm.
   c. After vortexing, the samples were centrifuged at 2000 x g.
   d. Supernatant for each precipitation was transferred to injection vials and diluted with 200µl of water.
7. 15µl of each sample was injected into an AB Sciex API-4000 triple quadrapole mass spectrometer for analysis.

### Frozen Aliquotting Precision Evaluation

1. Nisoldepine and MABG were observed to be fairly stable due to the low CVs across $T_0$-$T_4$ and were used as a gauge for frozen aliquotting precision.
2. Because freeze-thaw aliquots were generated in the liquid form (post-thaw), frozen aliquotting precision can be considered in comparison to liquid aliquotting precision.

### Compound Stabilization Evaluation

1. The stability of a given compound was judged by the recovery observed at the last time point ($T_4$) in relation to the baseline recovery ($T_0$).

$$Recovery = 1 - \frac{(T_0 - T_4)}{T} = 100\%$$

2. Except for the use of EDTA plasma, no other chemical stabilizers were utilized.

[1] Li W, Zhang J, Tse FLS. Strategies in quantitative LC-MS/MS analysis of unstable small molecules in biological matrices. Biomed. Chromatogr. 2011; 25: 258–277.