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

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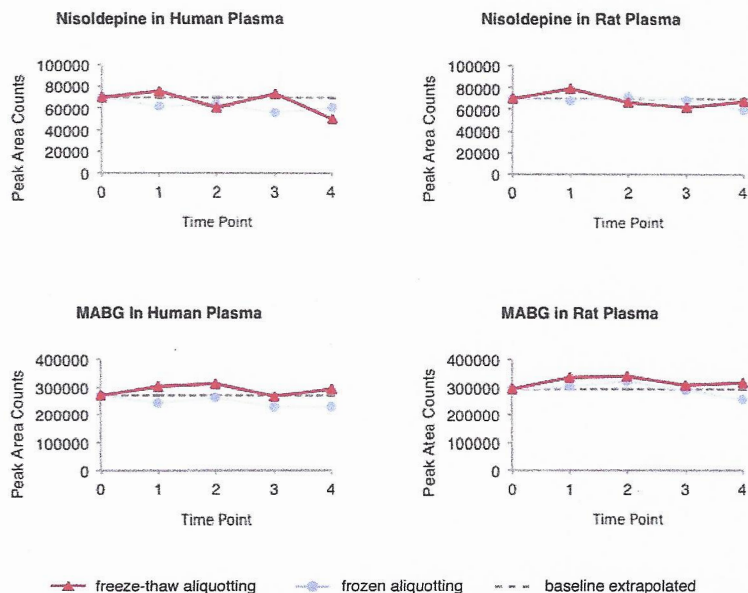
Introduction

Drug development and pharmaceutical research relies heavily on animal and human bio-specimens to generate the vast amounts of 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. Furthermore, it is not uncommon for the development time lines of bioanalytical assays to be extended by weeks, or even months, as a result of efforts to identify stabilization protocols for the target compounds. The CXT 750 Frozen Sample Aliquotter, an automated instrument commercialized by CryoXtract Instruments, can enable an intact cold chain work-flow for bioanalysis of freeze-thaw sensitive compounds and bio-specimens such as plasma. By employing a novel technology known as frozen aliquotting, aliquots of frozen bio-specimens generated on the CXT 750 can be directly inputted into sample preparation procedures, allowing for the simultaneous thawing and stabilization of the target compounds prior to analytical procedures. The following data was generated in collaboration with GlaxoSmithKline (GSK) and demonstrates the improvements frozen aliquotting can provide for compound stabilization and bioanalysis of small molecule compounds and peptides.

Experimental Overview and Results

A test mixture consisting of varying degrees of labile small molecule compounds and peptides (see Test Compounds on page 2) was spiked into human and rat EDTA plasma samples at a single concentration. After an initial baseline recovery for each compound was determined by LC-MS/MS, the human and rat test mixtures were frozen in individual cryogenic vials and then subjected to four rounds of freeze-thaw aliquotting and frozen aliquotting, the latter being performed on the CXT 750. Aliquots generated at each round were treated to a protein precipitation step and then again analyzed by LC-MS/MS. For each compound in the human and rat test mixtures, the analytical recovery was determined using the peak area counts represented in the individual chromatograms.

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



Experimental Methodology

1. Frozen samples were prepared as 1.8ml aliquots in 2.0ml cryogenic vials and frozen in a -80°C freezer.

2. Each aliquotting condition (freeze-thaw and frozen aliquotting) consisted of three frozen vials of spiked rat EDTA plasma and spiked human EDTA plasma.

3. 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.

4. For each round of frozen aliquotting, a single core (frozen aliquot), 100µL in volume, was generated per sample on the CXT 750. A total of four cores were removed from each frozen aliquotted sample over the course of the study.

5. Samples were prepped for LC-MS/MS analysis by performing a protein precipitation step using 1:1 acetonitrile to methanol.

6. Frozen aliquots (100µL) were added to the protein precipitation step directly and allowed to thaw in the acetonitrile/methanol mixture. Aliquots (100µL) from the freeze-thaw condition were added in liquid form.

Frozen Aliquotting Precision

- Compounds observed to have fairly low CVs from time point zero through time point four were considered to be stable in response to the experimental conditions.

- Because freeze-thaw aliquots were generated in the liquid form (post-thaw), frozen aliquotting precision is being compared to liquid aliquotting precision in this study.

- Frozen aliquotting precision, as judged by the analytical recovery of nisoldepine and MABG, was observed to be comparable to liquid aliquotting and also corresponds with the volumetric performance of the CXT 750 (5-10% CV).

Table 1: Summary of LC-MS/MS Recovery CVs for T0-T4

	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%

Compound Stabilization

- The stability of a given compound was judged by the recovery observed at the last time point (T4) in relation to the baseline recovery (T0).

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

- Besides the use of EDTA-treated Plasma, no other chemical stabilizers were utilized in this study.
- Frozen aliquotting was observed to result in significantly improved recoveries by LC-MS/MS for both human and rat plasma for compounds shown to be susceptible to degradation during typical freeze-thaw procedures (see Figure 2 and Table 2).

Table 2: Summary of Percent Recoveries at T4

	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%

Test Compounds

Nisoldepine – Small molecule drug susceptible to photochemical degradation.[1]

MABG - Mycophenolic acid beta glucoronide; Antibiotic metabolite susceptible to degradation at physiological pH.[1]

Angiotensin I – Peptide hormone susceptible to enzymatic degradation.

Caffeic Acid – Naturally derived organic compound susceptible to esterase-induced degradation.[1]

Cisatracurium – Small molecule drug (susceptible to esterase mediated hydrolysis.[1]

References

[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

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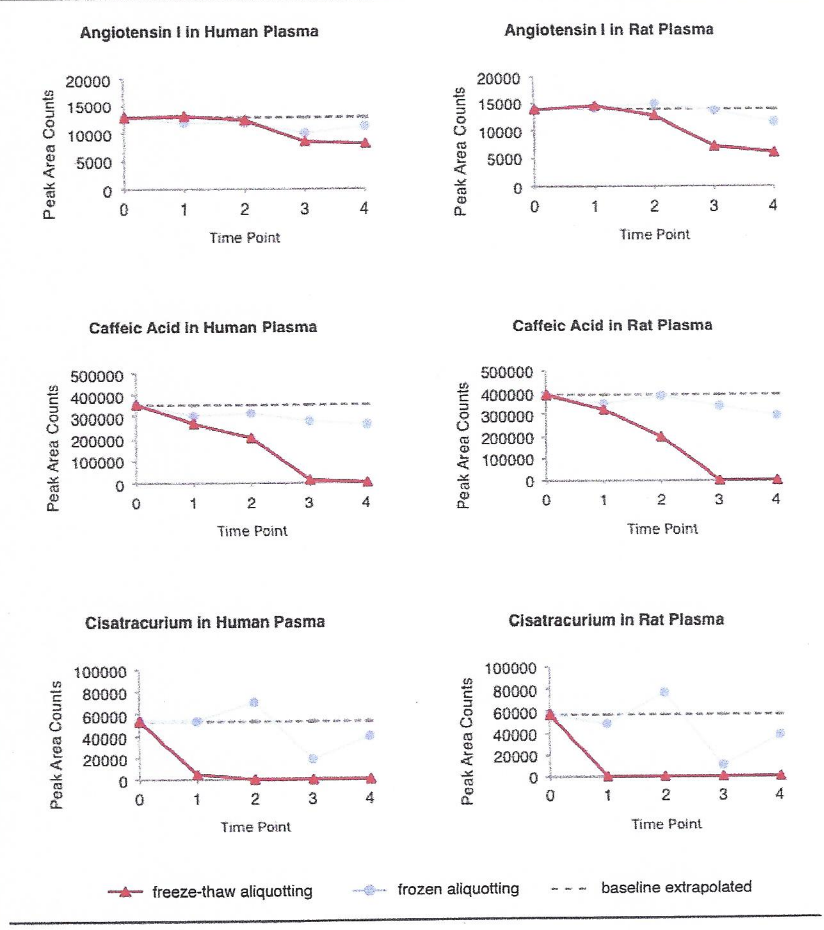
Peak area counts for nisoldepine and MABG were observed to be fairly stable across all time points (T0 – T4) for both rat and human samples. CVs ranged from 6.1 – 22.8% for freeze-thaw aliquots. For frozen aliquots produced on the CXT 750, CVs were observed to be below 10% (7.6 – 9.6%) for the same compounds (see Figure 1 and Table 1). In other evaluations not reported in this document, the precision of the CXT 750 in regards to generating frozen aliquots of a specific volume is about 5 -10% CV. Based on this and the observed analytical CVs for nisoldepine and MABG, frozen aliquotting was observed, in this study, to be a quantitative method for aliquotting small molecule compounds in frozen EDTA plasma.

Angiotensin I, caffeic acid, and cisatracurium all showed a marked decline in peak area counts across time points zero through four for freeze-thaw aliquots (see Figure 2 and Table 2). However, significant improvements in stability were observed when frozen human and rat plasma samples were aliquotted using the CXT 750. Notably, caffeic acid showed essentially zero recovery by LC-MS/MS after two to three freeze-thaw cycles whereas 75% of the compound remained recoverable, in both human and rat plasma, after four frozen aliquotting rounds. The most dramatic impact was observed for cisatracurium in which little to no detectable amounts were observed by LC-MS/MS after a single freeze-thaw cycle. Though the recovery response was observed to be variable, it is clear that frozen aliquotting was a key factor for detection of cisatracurium by LC-MS/MS in this study.

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



Conclusions

In this study, 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 were 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.