



Mouse Clinical Pathology: Clinical Chemistry Controlling Variables that Influence Data



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ABSTRACT Relatively simple and inexpensive clinical chemistry tests can provide valuable data relevant to phenotyping and other studies involving rodents. Clinical chemistry (serum or plasma biochemistry) tests can contribute information relevant to various phenotypes including but not limited to liver function, kidney function, endocrine abnormalities and lipid metabolism.

Small specimen volumes, and survival collections from mice are a special challenge, and require prioritization of tests. Animal handling variables as well as specimen collection, handling, storage and processing impact data, and can result in excessive variability and poor reproducibility, and in artifacts that can be misinterpreted as phenotypes, or otherwise significant findings. Specific issues in animal handling, specimen collection, handling, storage and processing were investigated. Collection sites, storage temperature, hemolysis, delay to processing, and collection tubes are some factors that influence data generated by automated clinical chemistry instruments, and ultimately influence published data, statistical analysis, interpretation and conclusions.

To optimize the utility of relatively inexpensive automated clinical chemistry equipment for our phenotyping core clinical pathology laboratory, we assessed the impact of these factors using our VetAce® (Alpha Wasserman, West Caldwell, NJ) clinical chemistry analyzer, which can perform a range of clinical chemistry panels on 100-300ul of serum or plasma. Terminal specimen collections of mice and rats designated for euthanasia were obtained at euthanasia from barrier-raised and -maintained animals of various stocks, strains, sexes and ages. Based on our results, we developed recommendations for our facility and equipment, for prioritizing tests to be conducted on small blood volumes, as well as for standardizing mouse blood collection and handling to minimize clinical chemistry variables that can result in 'false phenotypes'.

INTRODUCTION Blood parameters play a critical role in diagnosis, assessing progression, and characterization of disease and phenotypes in clinical and research situations. The accuracy and reliability of the data depend on identification and control or elimination of variables that may affect these results. Different blood collection and handling strategies represent a source of variability that can be controlled in many instances.

To develop blood collection and handling recommendations for our facility and equipment, we investigated factors that were recognized to vary among submissions to our laboratory, and might influence data derived from the VetACE® clinical chemistry analyzer. These included **collection sites and methods, hemolysis, tube/additive selection, delay to processing, and repeated freezing and thawing.**

STUDY DESIGN Animal care and use was in compliance with the Guide¹ and approved by Johns Hopkins Institutional Animal Care and Use Committee (IACUC). The mice were adult male C57BL/6 and the rats were adult female Sprague Dawley. Mice were manually restrained for facial blood collection and a 20g needle (Terumo®) was used as a lancet. The mice were then euthanized with CO₂ inhalation for cardiac blood with via a 21g needle (Terumo®) and 1 ml syringe (BD). The rats were euthanized with CO₂ inhalation for caudal vena caval bleed via a 20 g needle (Terumo®) and 10 ml syringe (BD®). Bleeding was performed cage side to limit travel stress, and to parallel research practices in our barrier facility. The 5 specimen collection / handling strategies tested are summarized here.

Collection Site and Method:

Species: Mouse **Site:** Facial; Cardiac Open, Closed, n = 10

Tubes: Microtainer® serum separator tubes SST (#365956)

'Normal' Control: Facial vessel drip into SST, allowed to clot, spun, and serum processed immediately

Treatment variable: Site and method: Closed (blind) cardiocentesis, VS open cardiocentesis with heart visualized and blood drawn from right ventricle. (21g needle /1ml syringe)

Hemolysis

Species: Rat **Site:** Caudal vena cava n = 10

Tubes: BD Vacutainer® no additive tube (#367812)

'Normal' Control: Blood placed in a no additive tube (1), allowed to clot, spun and serum analyzed immediately

Treatment variable: Hemolysis: Tube 2 blood vortexed x 5 seconds. Tube 3 (positive control) hemolyzed by addition of 50uL deionized. The blood was allowed to clot, spun and analyzed immediately.

Tube/Additive:

Species: Rat **Site:** Caudal vena cava n = 10

Tubes: Whole blood collected and aliquoted into 5 tubes:

1. Microtainer® serum separator tubes SST (#365956);
2. Microtainer® lithium heparin coated tube (#365965);
3. Microtainer® plasma separator tube (#365985),
4. Microtainer® no additive tube (#365963)
5. no additive tube + 50ul sodium heparin.

'Normal' Control: Blood in no additive tube, allowed to clot, spun and serum analyzed immediately

Treatment variable : Different tubes/additives:

Specimens allowed to clot, spun, then serum or plasma analyzed immediately.

Delay to Processing/analysis

Species: Mouse **Site:** Cardiac n = 10

Tubes: Microtainer® SST (#365956). Whole blood collected and divided equally into 2 SST.

'Normal' Control: Blood in SST, allowed to clot, spun and serum analyzed immediately

Treatment: Delay: Blood in SST x 24 hr, then spun & serum analyzed.

Freeze/Thaw

Species: Rat **Site:** Caudal vena cava n = 10

Tubes: Whole blood collected in 6ml no additive tube (#367815), allowed to clot, spun, and serum divided into 7 Microtainer® no additive tubes (#365963).

'Normal' Control: Blood in no additive tube, allowed to clot, spun and, serum analyzed immediately

Treatment: Freeze/Thaw: Serum frozen at -20°C or -80°C x 24 hr, thawed x 24 hr, refrozen x 24 hr, for 2 cycles, 4 cycles, or 6 cycles.

Statistics: Excel Mean, Standard Deviation and Student T-test- 2 tailed-paired.

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RESULTS All samples were analyzed using a VetAce® (Alpha Wasserman, West Caldwell, NJ). A 15 parameter profile was performed, including cholesterol, high-density lipoprotein (HDL), lactate dehydrogenase (LDH), triglycerides, creatine kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), glucose, albumin, total protein, calcium, blood urea nitrogen (BUN), creatinine and amylase. Electrolytes require at least 250 ul of serum. If specimen was sufficient, sodium, potassium and chloride were included.

Collection Site & Method: *Small but statistically significant differences:*

- ▲ Higher creatine kinase, AST and glucose from both open and closed cardiac puncture cardiocentesis compared to facial vessels.
- ▲ Creatine kinase and glucose higher in the open vs the closed cardiac puncture.

Hemolysis: Statistically significant differences were *not* detected when comparing baseline to samples with mild hemolysis. *Small but statistically significant differences:*

- ▲ Higher LDH in samples with moderate to severe hemolysis compared to non-hemolyzed baseline specimens.
- ▼ Lower sodium and chloride in samples with moderate to severe hemolysis compared to non-hemolyzed baseline specimens

Tube Selection: Statistically significant differences were *not* detected between the plain tube and the serum separator (gel) tube.

Small but statistically significant differences:

- ▼ Lower triglycerides, creatine kinase and LDH in lithium heparin, sodium heparin and plasma separator gel tube compared to plain (no additive) tubes.

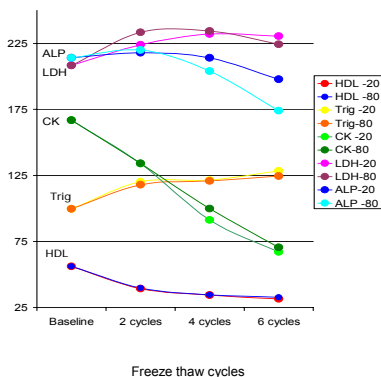
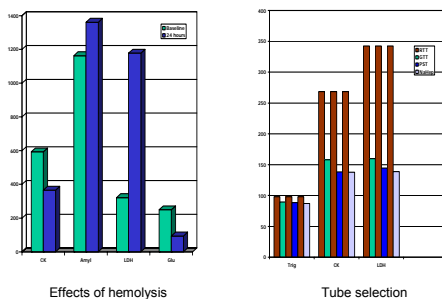
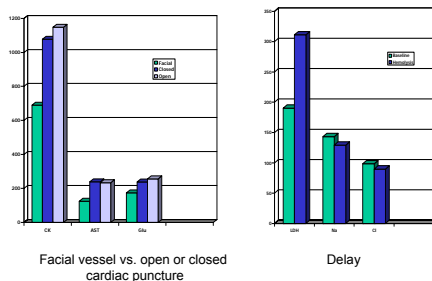
Delay to processing: *Small but statistically significant differences:*

- ▲ Higher cholesterol, triglycerides, amylase, and LDH in samples allowed to sit on the benchtop for 24 hours after collection then separated and analyzed compared to samples analyzed immediately after collection and separation (<1 hour).
- ▼ Lower creatine kinase, AST, glucose, total protein, calcium and creatinine in samples allowed to sit on the benchtop for 24 hours after collection then separated, compared to sample analyzed immediately after collection and separation (<1 hour).

	Chol	Trig	AST	Glu	TP	Ca	Creat
Baseline	132.7	126.3	138.2	249.3	8.255	10.715	0.35
24 hours	154.5	135.9	105.75	93.95	6.66	9.48	0.16

Freeze/Thaw: *Small but statistically significant differences:*

- ▲ Higher triglycerides and LDH in all frozen/thawed specimens, compared to the same samples analyzed immediately after collection/separation (<1 hour). The values continued to rise as more freeze thaw cycles were performed.
- ▼ Lower HDL and creatine kinase in all frozen/thawed specimens, compared to the same samples analyzed immediately after collection/separation (<1 hour). The values continued to fall as more freeze thaw cycles were performed
- ▼ Lower alkaline phosphatase on the 6th freeze thaw cycle.



DISCUSSION/RECOMMENDATIONS Statistically significant differences were achieved by common variations in specimen handling seen in submissions to our laboratory. While these differences may have little or no significance in a clinical setting, they could be expected to complicate data analysis in a research setting. These represent only a few variations in specimen collection and handling that could be expected to influence chemistry results, but they emphasize the importance of consistent specimen collection and handling practices within and between studies.

Collection Site and Method:

- ▶ The same blood collection site and method should be used for comparisons within and between studies.

Higher serum levels of muscle enzymes with cardiocentesis, compared to facial vessel bleeding, are presumed to be due to unavoidable trauma to skeletal and heart muscle during cardiocentesis. As expected the differences were greater with the 'open' (exposed heart) technique. Such differences should be expected to vary with expertise of the operator.

Hemolysis:

- ▶ Minimize hemolysis by gentle handling of blood specimens.

Although significant changes were not determined to result from mild hemolysis in this small study, grossly evident, moderate to severe hemolysis did cause statistically significant changes. Increased serum potassium levels, due to release of intracellular potassium in hemolyzed specimens, was expected, but was not detected in this small study using rat blood. This finding may be related to our small sample size, species differences in intracellular potassium, erythrocyte fragility, or other factors²

Tube Selection:

- ▶ The same type of blood collection tube should be used for comparisons within and between studies.

When spun and separated promptly (< 60 min), differences were not detected between results from plain and serum separator (gel) tubes. Although gel tubes are expected to confer an advantage when prolonged holding of unseparated blood specimens cannot be avoided, this was not tested specifically. Gel tubes also may confer an advantage in obtaining 'clean' (cell-free) serum or plasma from small specimens (e.g. from mice). Lithium heparin anticoagulant tubes are useful when hematology (CBC) evaluations are to be performed on the same specimen, but there may be statistically significant differences between results from plasma compared to serum. When liquid anticoagulants are used a dilution effect on small blood specimen should be considered

Delay to Processing:

- ▶ Time delay to processing should be minimized, especially when glucose is an important parameter.

Processing delays often are unavoidable; however delay in separating the serum from the clot can result in statistically significant changes in some parameters. Glucose diminished most significantly, as should be expected with access to metabolically active cells. When glucose is an important parameter, use of separator (gel) tubes, and/or glucose tubes (with sodium fluoride) should be considered.

Freezing and Thawing:

- ▶ Freeze / thaw cycles should be minimized.

Most parameters were not detectably affected by freezing of serum, but repeated freeze thaw cycles resulted in statistically significant differences in some parameters.