Unrestricted Document

Context-of-Use Evaluation of a Simplified LC-MS/MS Assay for Endogenous NAD in Rodent Tissue

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INTRODUCTION

▶Nicotinamide adenine dinucleotide (NAD) is a widely used endogenous biomarker of metabolic and mitochondrial health, but it is enzymatically and thermally labile—rapid ex vivo degradation is a major risk to accurate tissue quantitation.[1]

▶Published LC-MS/MS methods for tissue NAD are often complex, combining multiple enzymatic and chemical control steps that are difficult to translate into routine, high-throughput practice.[1-3]

▶Early discovery DMTA cycles run in parallel and need NAD data quickly to inform decisions, so the method had to be fast, simple, and low-cost per sample.

▶We therefore simplified this down to two core principles—continuous temperature control and enzymatic inhibition[1]—and built a practical cryolysis-based[2] workflow around them.

▶We evaluated the method through a Context-of-Use (CoU) lens appropriate to its intended application: discovery-level comparison of treated and control tissue, where our internal discovery guidance applies a 30% acceptance criterion rather than full regulatory validation.

QUESTION

Does NAD in treated tissue differ from concurrent controls, and by how much?

DECISION

Rank compounds and groups within single studies, to inform discovery decisions

NOT FOR

Absolute levels, or comparison across studies, occasions or vehicles

MATERIALS & METHODS

1 Homogenise — Cryolysis into HALT inhibitor + EDTA buffer

2 Precipitate — Internal standard added; all steps on wet ice

3 Isolate — Centrifuge at 4°C; transfer supernatant

4 Quantify — LC-MS/MS vs. a same-day calibration curve

SAMPLE PREPARATION	
Homogenization	Precellys® Cryolys®, 1:3 tissue:buffer; 5500 rpm, 2 cycles of 10 s on / 20 s off, 0°C
Buffer	PBS (~pH 7.4) with 1% Halt™ protease/phosphatase inhibitor cocktail + 5 mM EDTA
Matrix	20 µL tissue homogenate; calibrators/QCs in surrogate matrix (2% BSA in 0.1 M PBS + 1% inhibitor cocktail)[3,4]
Precipitation	3:1 methanol + 0.1% formic acid containing IS (60 µL to 20 µL)
Internal standard	Metoprolol, 1 µM (100 µL of 10 mM stock in 1 L precipitant)
Centrifugation	4000 rpm, 4°C: 10 min, re-mix, then 5 min
Final dilution	50 µL supernatant + 50 µL water
Temperature	Wet ice throughout; autosampler 15°C

LC-MS/MS	
System	Xevo® TQS Micro with Premier LC System
Column	ACQUITY® Premier HSS T3, 1.7 µm, 2.1 × 50 mm, 40°C
Mobile phase	A: 10 mM ammonium acetate (aq); B: methanol
Gradient	1.65 min, 1-99% B at 0.4 mL/min
Injection	10 µL
Detection	ESI+, MRM 664.0 > 136.0 (NAD)
Calibration	Same-day curve, 20-20,000 ng/mL (80-80,000 ng/g tissue)
Application	Liver and heart, multiple studies, 5 occasions over ~15 months

CASE STUDY: STUDY DESIGN

Vehicle 1 (Formulation 1) → **Compound X** — 1x dose — Different compound, not in the A-D series

Not directly comparable to A-D — Different compound and different dose

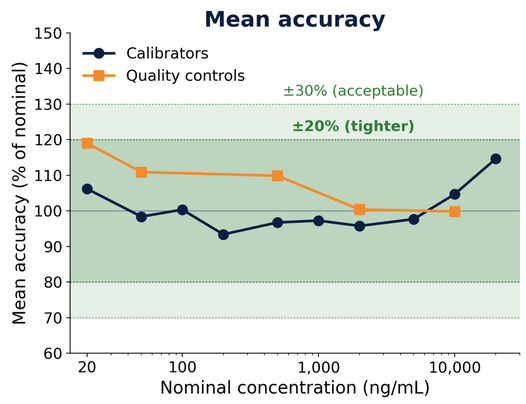
Vehicle 2 (Formulation 2) → **Compound A** 3x dose same series; **Compound B** 3x dose same series; **Compound C** 3x dose same series; **Compound D** 3x dose same series

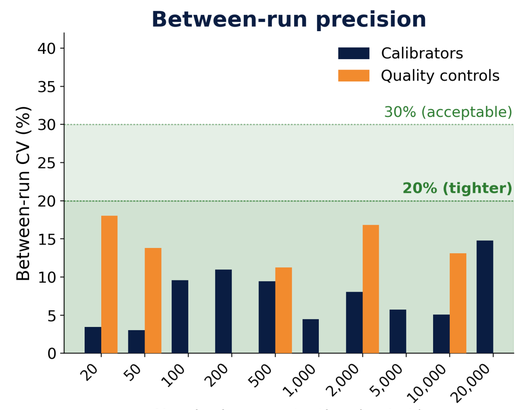
Doses are mg/kg relative to Compound X (1x).

▶Single pharmacology study analyzed on one occasion (27-Aug-2026); liver and heart; n = 5 animals per group (n = 4 for two liver groups after exclusion of statistical outliers by Grubbs' test). Compounds A-D are analogs from one series; Compound X is a different compound.

▶No prior expectation of compound effects on NAD was available at the time of analysis, so the interpretation presented here is unbiased by expectation.

RESULTS: METHOD PERFORMANCE





▶Mean accuracy and between-run precision for each calibrator level (4-6 runs) and QC level (triplicate per run; 9-18 replicates from 3-6 runs), pooled across runs analyzed over ~15 months.

▶Every run met the acceptance criteria of our internal discovery guidance (≥75% of calibrators and ≥67% of QCs, with ≥50% at each QC level, within ±30%). Pooled accuracy was within ±20% and CV ≤19% at every level (20-20,000 ng/mL).

▶Stability: published data show NAD+ losing up to 50% in 10 min in room-temperature blood and ~3-4% on wet ice, but remaining stable at -80°C for ≥3 weeks[4]. In this work, re-analysis of control liver homogenate after -80°C storage and freeze-thaw agreed within ±7% of the original result; QCs analyzed late in runs also met criteria.

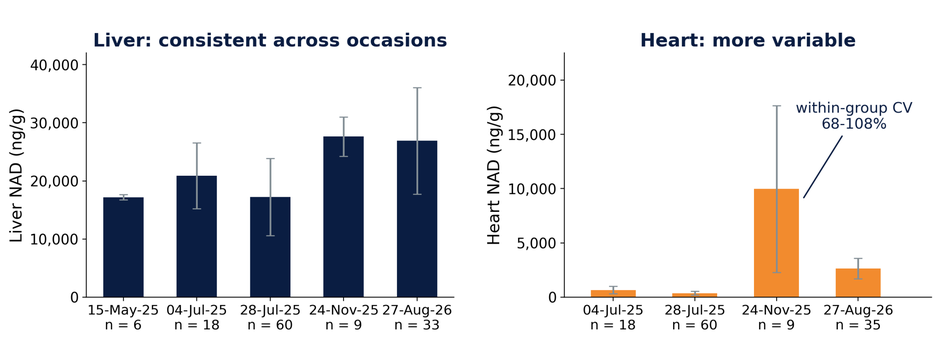
Designed for discovery

Surrogate matrix — No blank tissue exists for an endogenous analyte, so calibrators/QCs use BSA/PBS

Analogue IS — Metoprolol, typical of rapid discovery bioanalysis

Targeted checks — Stability tested on stored homogenate; no formal validation

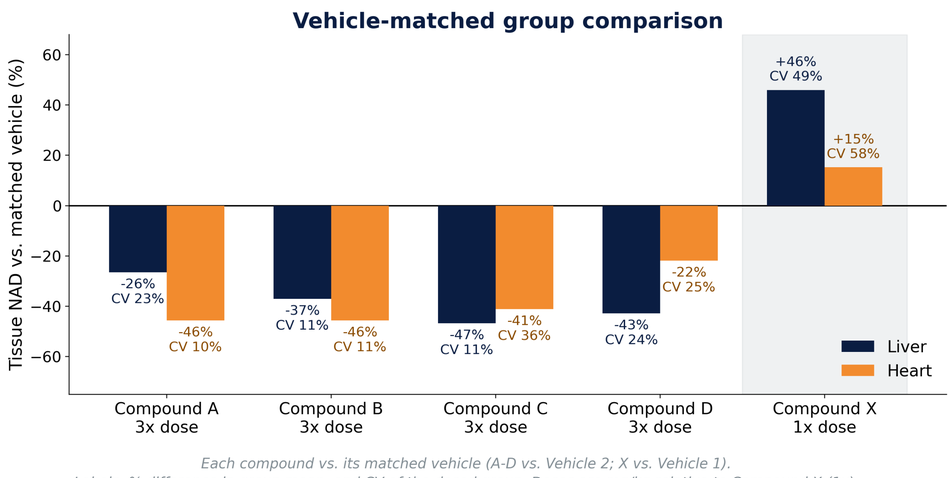
RESULTS: BETWEEN-OCCASION VARIABILITY



Inter-study CVs of ~50-75% for NAD(H) in mouse liver have been reported[5]

▶Mean ± SD of all samples per occasion; study designs and sample numbers differed. Liver means spanned 1.6-fold and heart means 7-fold (27-fold with 24-Nov-2025, when heart was also highly variable within groups, CV 68-108%, but liver from the same animals was not, CV 9-17%, and QCs passed).

CASE STUDY: VEHICLE-MATCHED RESULTS



Each compound vs. its matched vehicle (A-D vs. Vehicle 2; X vs. Vehicle 1).
Labels: % difference in group mean, and CV of the dosed group. Doses are mg/kg relative to Compound X (1x).

▶Single study analyzed on one occasion (27-Aug-2026). Vehicle groups differed at baseline (Vehicle 2 vs. 1: +49% liver, +86% heart), so each compound is compared with its own vehicle. Compounds A-D (3x dose) gave lower NAD than matched vehicle in both tissues (-22% to -47%), larger than the assay's imprecision (CV ≤19%); Compound X (1x dose) showed no reduction (CV 49-58%).

SUMMARY

▶A simplified, cold-chain, enzyme-inhibited LC-MS/MS workflow, built to inform discovery decisions, met acceptance criteria in every run over ~15 months, for a context of use of relative comparison with concurrent controls.

▶Absolute NAD varied with tissue, occasion and vehicle (liver consistent across occasions; heart more variable, with one occasion markedly higher; vehicle baselines +49% liver, +86% heart), in line with published inter-study variability[5], so interpretation relies on same-study, vehicle-matched controls.

▶In the case study, reductions of -22% to -47% for four analogs (3x dose) exceeded assay imprecision (CV ≤19%) in liver and heart, while a different compound (X, 1x dose) showed no reduction.

REFERENCES

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