Quantitative measurement of near full-length dystrophin and muscle content normalizer proteins in human muscle by IA-UPLC-MS/MS

Poster # 276P

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Introduction

- Accurate measurement of dystrophin protein in muscle tissue is crucial for developing new therapies for Duchenne muscular dystrophy (DMD)
- Historically, western blot (WB) methods have been used for semi-quantitative measurement of dystrophin in muscle biopsies[1]
- WB methods have difficulty measuring very low dystrophin concentrations and are prone to high variability[2]

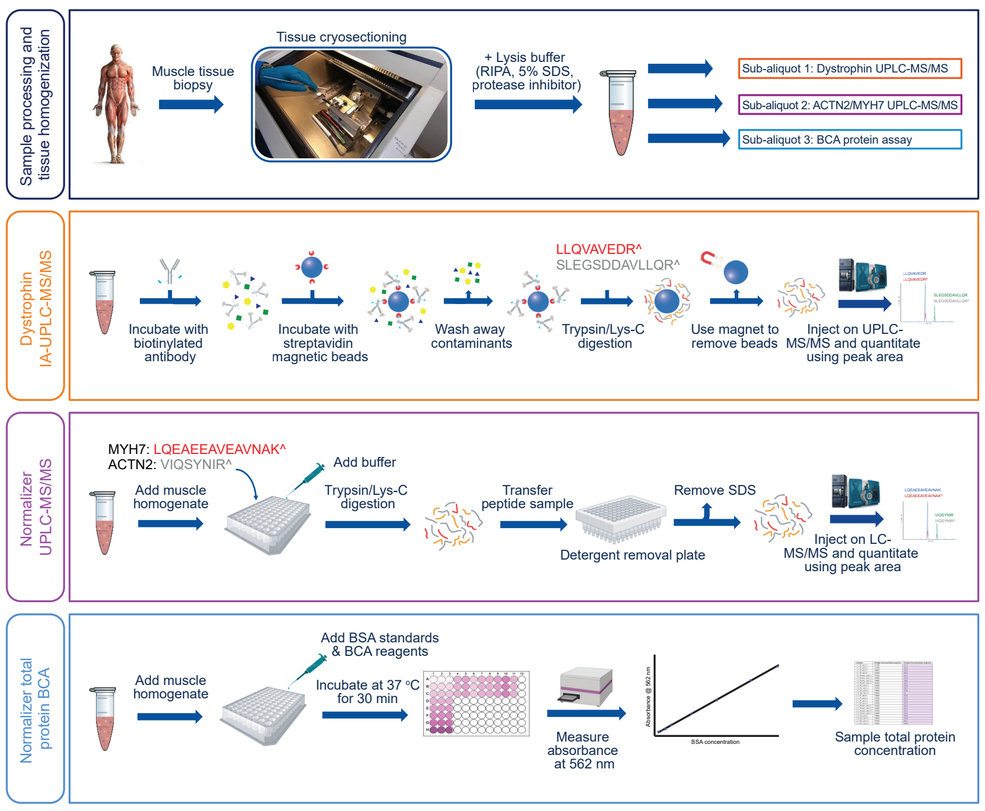
Objective

- We developed a sensitive, precise, accurate, and reproducible immunoaffinity ultra-performance liquid chromatography coupled with tandem mass spectrometry (IA-UPLC-MS/MS) method to measure the absolute concentration of near full-length dystrophin protein in human muscle tissue and companion methods to measure muscle content normalizer proteins (alpha-actinin-2 [ACTN2] and β-myosin heavy chain [MYH7]) and total protein

Methods

- Muscle biopsy samples were cryosectioned (10 µm) and homogenized, and lysate aliquots were used to measure dystrophin, muscle normalizer proteins, and total protein (**Figure 1**)

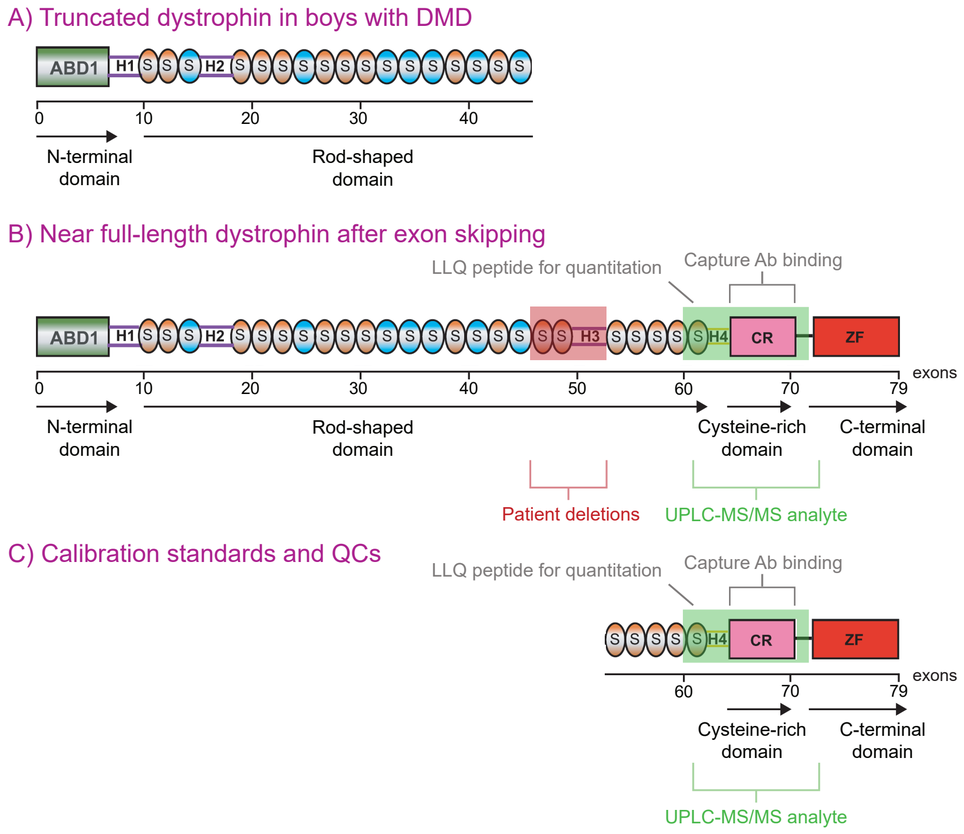
Figure 1. Dystrophin and muscle normalizer assay workflow



^Denotes heavy isotope-labelled internal standard peptides that are added to samples prior to digestion.
ACTN2, alpha-actinin-2; BCA, bicinchoninic acid assay; BSA, bovine serum albumin assay; IA, immunoaffinity; UPLC-MS/MS, ultra-performance liquid chromatography coupled with tandem mass spectrometry; MYH7, β-myosin heavy chain; RIPA, radioimmunoprecipitation assay buffer; SDS, sodium dodecyl sulfate.

- Calibration standards and quality control samples to monitor assay performance were prepared by adding a recombinant 140-kDa dystrophin isoform (Dp140) to blank dog muscle homogenate (**Figure 2**)
  - For the dystrophin UPLC-MS/MS assay, 2 peptides were monitored. LLQVAVEDR (LLQ) was used for quantitation because it showed a better signal:noise ratio, and SLEGSDDAVLLQR (SLE) was monitored to confirm analyte identity

Figure 2. Schematic of UPLC-MS/MS analytes



Ab, antibody; ABD, actin-binding domain; CR, cysteine-rich domain; DMD, Duchenne muscular dystrophy; H, hinge region; LLQ, LLQVAVEDR; QC, quality control; S, spectrin; UPLC-MS/MS, ultra-performance liquid chromatography coupled with tandem mass spectrometry; ZF, zinc-finger domain.
Figure 2 reproduced with minor changes from Wang Y, et al. Prenatal diagnosis of Duchenne muscular dystrophy revealed a novel mosaic mutation in Dystrophin gene: a case report. *BMC Med Genet*. 2020 Nov 11;21(1):222. Licensed under CC BY 4.0 and cropped from original.

Results

- The dystrophin UPLC-MS/MS method was validated in compliance with the current International Council for Harmonization (ICH) M10 and US Food and Drug Administration assay validation guidance (**Table 1**)

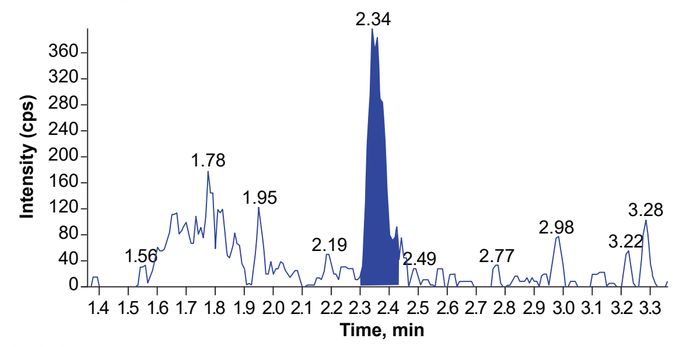
Table 1. Validation of the dystrophin UPLC-MS/MS method

Parameter	Validation result
Inter-assay precision	<20% CV (<25% CV at LLOQ)
Inter-assay accuracy	<20% Bias (<25% bias at LLOQ)
Limit of quantitation	100 pM (~0.9% of normal)
Limit of detection	<60.0 pM (lowest measured ~23 pM; 0.26% of normal)
Sample long term stability	>7 months

Additional parameters validated for the UPLC-MS/MS assay include precision and accuracy of calibrators and quality control samples, range of quantitation, limit of detection, carryover, parallelism, matrix effect, recovery, freeze/thaw and short-term analyte stability, reinjection and processed sample stability, intermediate solution stability, and sample long-term stability (ongoing).
CV, coefficient of variation; LLOQ, lower limit of quantitation; UPLC-MS/MS, ultra-performance liquid chromatography coupled with tandem mass spectrometry.

- Dystrophin analyte peaks were detectable in muscle samples from untreated boys with DMD at concentrations as low as 23 pM (~0.26% of normal; **Figure 3**)

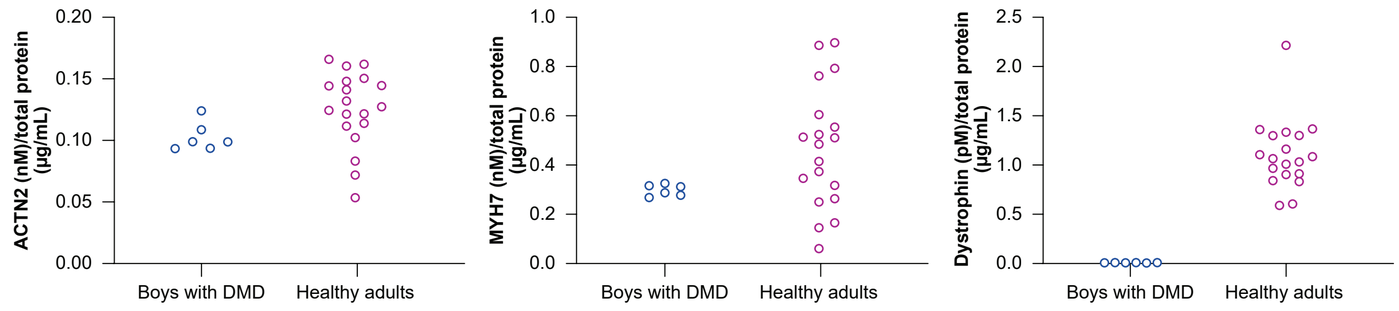
Figure 3. Sensitive detection of near full-length dystrophin in DMD muscle



DMD, Duchenne muscular dystrophy.

- The absolute dystrophin, ACTN2, and MYH7 concentrations in 19 healthy adult muscle samples were used as a reference for reporting % of normal dystrophin concentrations in 6 muscle samples from boys with DMD (**Figure 4**)

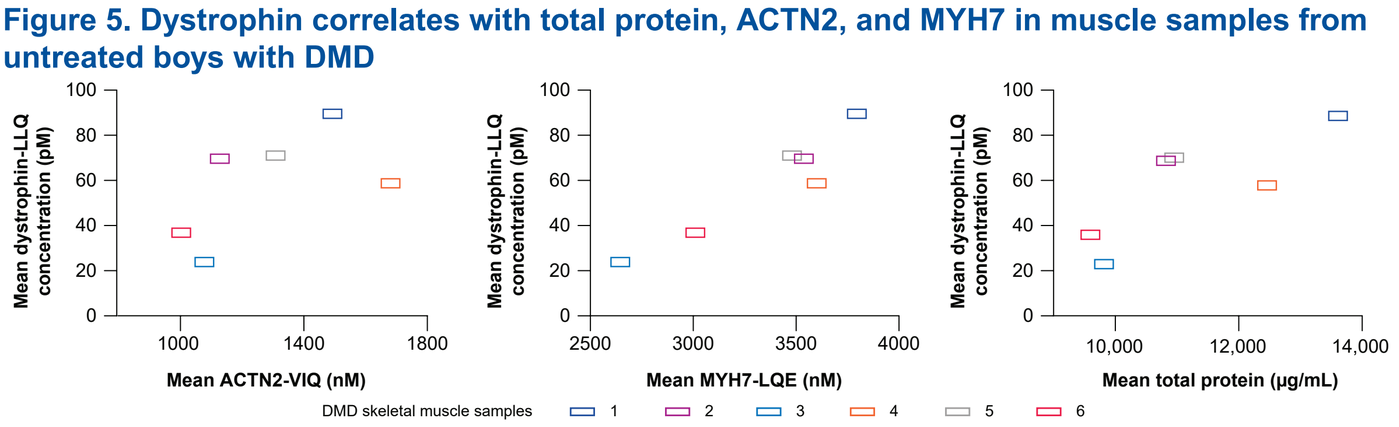
Figure 4. Dystrophin and normalizer protein concentrations in DMD and healthy human muscle



ACTN2, alpha-actinin-2; DMD, Duchenne muscular dystrophy; MYH7, β-myosin heavy chain.

- Dystrophin concentrations in muscle samples from untreated boys with DMD correlated with both total protein and ACTN2 or MYH7 concentrations (**Figure 5**)

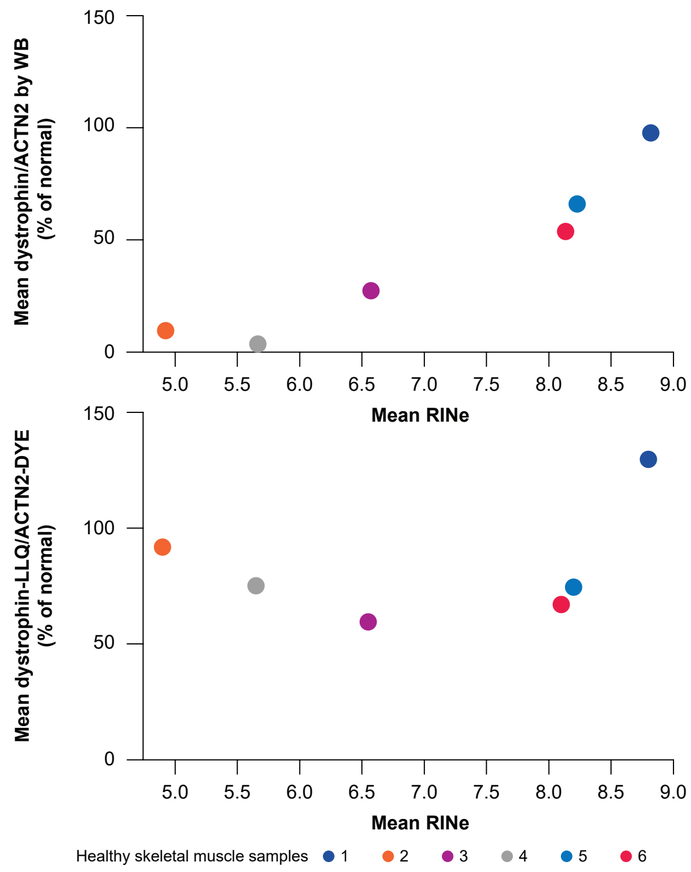
Figure 5. Dystrophin correlates with total protein, ACTN2, and MYH7 in muscle samples from untreated boys with DMD



ACTN2, alpha-actinin-2; DMD, Duchenne muscular dystrophy; LLQ, LLQVAVEDR; LQE, LQEAEEAVEAVNAK; MYH7, β-myosin heavy chain; UPLC-MS/MS, ultra-performance liquid chromatography coupled with tandem mass spectrometry; VIQ; VIQSYNIR.

- Dystrophin was measured by UPLC-MS/MS and WB in 6 healthy muscle samples with a range of RIN values (RNA integrity; measure of sample quality). UPLC-MS/MS measured high levels of dystrophin in all samples, but by WB, only samples with high RIN values contained high dystrophin levels (**Figure 6**)

Figure 6. Dystrophin measurement by WB is more susceptible to suboptimal sample handling and storage conditions compared to UPLC-MS/MS



ACTN2, alpha-actinin-2; DMD, Duchenne muscular dystrophy; DYE; DYESASLTEVR; LLQ, LLQVAVEDR; RINe, RNA Integrity Number equivalent; WB, western blot; UPLC-MS/MS, ultra-performance liquid chromatography coupled with tandem mass spectrometry.

Conclusion

- We developed a sensitive, precise, and reproducible method for absolute quantitation of dystrophin protein in parallel with ACTN2 and MYH7 to enable accurate adjustment for differences in muscle content in human muscle biopsy samples
- The UPLC-MS/MS method is more precise than WB and is less susceptible to analyte loss during sample storage and processing
- In comparison to healthy adult muscle, muscle from boys with DMD had slightly lower ACTN2, similar to the slightly decreased muscle fiber content assessed by histology (not shown)
  - ACTN2 is expressed in all muscle fiber types and for this reason is preferred over MYH7 for normalization of dystrophin concentrations
- Muscle from boys with DMD had significantly lower MYH7 compared to healthy adults, potentially reflecting an age-related difference in the type I:type II fiber ratio in addition to disease-related decrease in muscle fiber content[3]
- The UPLC-MS/MS method can reliably detect dystrophin in pre-treatment biopsy samples from boys with DMD and quantitatively measure increased levels after treatment

References

1. Beekman C, et al. *PLoS One*. 2018;13(4):e0195850. **2.** Farrokhi V, et al. *Gene Ther*. 2022;29(10–11):608–15. **3.** Talbot J, et al. *Wiley Interdiscip Rev Dev Biol*. 2016;5(4):518–34.

Acknowledgements

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Disclosures

All authors are employees and stockholders of BioMarin Pharmaceutical Inc.

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