Measurement of Nitrate and Nitrite in Biopsy-Sized Muscle Samples

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Abstract

Studies of rats have demonstrated that skeletal muscle plays a central role in whole-body nitrate (NO_3^-)/nitrite (NO_2^-)/nitric oxide (NO) metabolism. The small size of human muscle biopsy samples, however, presents an analytical challenge in this context. Indeed, a recent study by Nyakayiru et al. (*J Appl Physiol* 2017; 123:637-644) reported that NO_2^- was below the limit of detection (LOD) using the "gold standard" chemiluminescent method even when assaying 40 mg of tissue.

PURPOSE To develop a method to precisely and accurately quantify the NO_3^- and NO_2^- content of biopsy-sized muscle samples.

METHODS NO_3^- and NO_2^- were extracted from rat soleus muscle samples using methanol combined with mechanical homogenization + ultrasound, bead beating, pulverization at liquid N_2 temperature, or pulverization + 0.5% Triton X-100. After centrifugation to remove precipitated proteins, NO_3^- and NO_2^- were measured using a dedicated high performance liquid chromatography analyzer with a LOD of <0.1 pmol.

RESULTS Mechanical homogenization + ultrasound resulted in the lowest NO_3^- content (62±20 pmol/mg), with high variability (CV >50%) across samples from the same muscle. The NO_2^-:NO_3^- ratio (0.019±0.006) was also elevated, suggestive of NO_3^- reduction during tissue processing. Bead beating or pulverization yielded lower NO_2^- and slightly higher NO_3^- levels, but reproducibility was still poor. Pulverization + 0.5% Triton X-100 provided the highest NO_3^- content (124±12 pmol/mg) and lowest NO_2^-:NO_3^- ratio (0.008±0.001), with the least variability (CV ~15%). These values are consistent with literature data from larger rat muscle samples analyzed using the chemiluminescent approach.

CONCLUSION We have developed a method capable of measuring NO_3^- and NO_2^- in muscle samples as small as 5-10 mg. The theoretical limit is even lower, i.e., 1 mg for both NO_3^- and NO_2^- or 5 ng for NO_3^- alone. This method should prove highly useful in investigating the role of skeletal muscle NO_3^-/NO_2^-/NO metabolism in both healthy and diseased subject populations, in response to exercise and dietary interventions, etc.

Introduction

It is now recognized that, rather than being biologically inert, nitrate (NO_3^-) can be a significant source of nitrite (NO_2^-) and hence nitric oxide (NO) in the body. Furthermore, studies of rats by Piknova and Schechter (1-3) have established that, at least in this species, skeletal muscle serves as an important reservoir of NO_3^- and NO_2^-, and hence a potentially important source of NO production, especially during contractile activity. Extending this research into humans, however, is challenging due to the low levels of NO_3^- and especially NO_2^- in muscle, combined with the small amounts of tissue available via muscle biopsy. Indeed, using the standard chemiluminescent method Nyakayiru et al. (4) were recently unable to detect NO_2^- in human muscle biopsy samples, despite assaying 40 mg of tissue.

In light of the above, the purpose of the present study was to develop and validate a high performance liquid chromatography (HPLC) method for quantifying muscle NO_3^- and NO_2^- in biopsy-sized muscle samples. Special attention was paid to maximizing NO_3^- yield and reproducibility while minimizing artifactual NO_2^- formation during tissue processing or storage.

Methods

- Male Sprague-Dawley rats were sacrificed and the soleus muscle rapidly excised and stored at -80°C until analysis.
- Duplicate samples weighing 5-10 mg each were cut from the mid-belly of each frozen muscle and NO_3^- and NO_2^- extracted using methanol combined with mechanical homogenization + ultrasound, bead beating, pulverization at liquid N_2 temperature, or pulverization + 0.5% Triton X-100 (n=4-14 samples/method).
- NO_3^- and NO_2^- were measured by injecting 10 uL of extract into a dedicated HPLC system (ENO-30, Eicom USA, San Diego, CA). In this system, NO_3^- and NO_2^- are first isolated from each other and from interfering substances on a separation column, NO_3^- reduced to NO_2^- on a cadmium column, then both reacted with Griess reagent before being detected spectrophotometrically at 540 nm.

Results

- The ENO-30 HPLC proved to be ideally suited for the present application, with excellent baseline stability (**Fig. 1**), sensitivity (limit of detection = 0.06±0.01 pmol; limit of quantification = 0.20±0.03 pmol; n=6 standard curves) (**Fig. 2**), and linearity of response (**Fig. 3**). A representative sample chromatogram is shown in **Fig. 4**.
- Pulverization of tissue at liquid N_2 temperature followed by extraction in 50 uL methanol + 0.5% Triton X-100 resulted in the highest NO_3^- and lowest NO_2^- contents and the least variability (**Figs. 5-7**). NO_3^- and NO_2^- values obtained using this approach were consistent with literature data from larger rat muscle samples analyzed using the chemiluminescent method (Ref. 1-3).
- NO_2^- content of tissue extracts stored at -80°C increased over time, suggesting residual xanthine oxidoreductase (XOR) activity (**Fig. 8**). Inclusion of 0.1 mmol/L oxypurinol in the extraction medium completed blocked this increase (**Fig. 8**).

Figure 1. Baseline stability of HPLC.

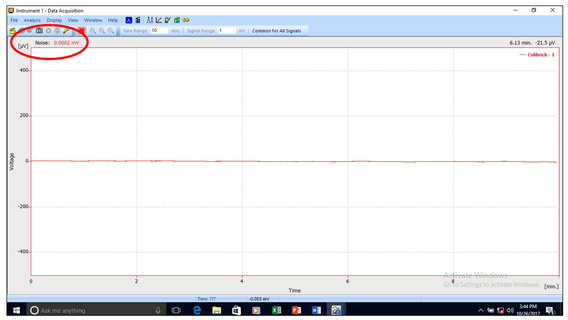


Figure 2. Sensitivity of HPLC (0.3 pmol NO_2^- standard).

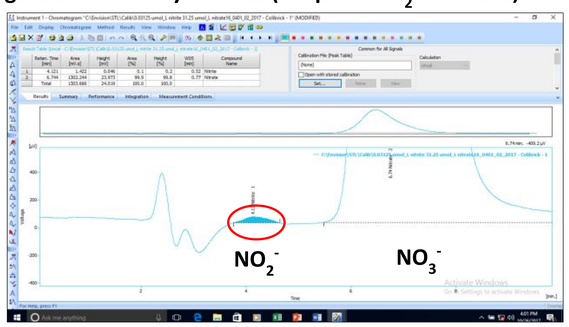
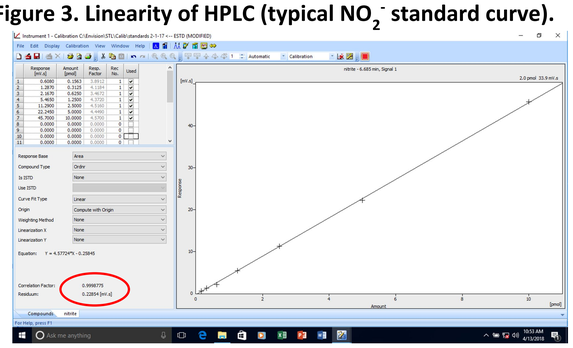


Figure 3. Linearity of HPLC (typical NO_2^- standard curve).



Results (con't)

Figure 4. Representative sample chromatogram

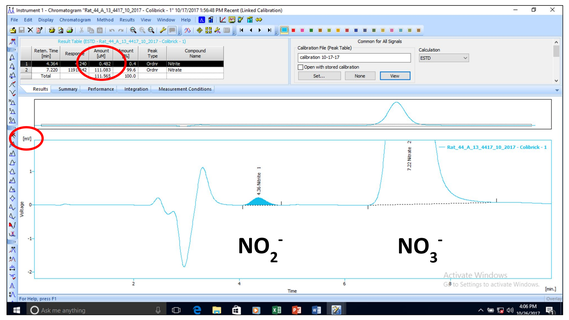


Figure 5. Effect of extraction method on NO_3^- content.

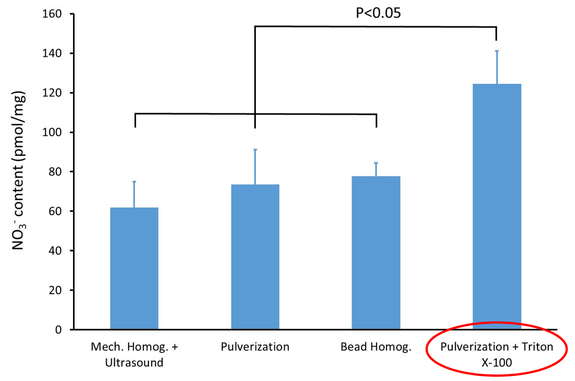
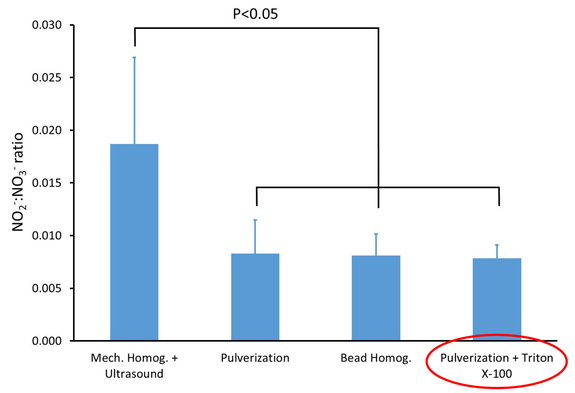


Figure 6. Effect of extraction method on NO_2^-:NO_3^- ratio.



Results (con't)

Figure 7. Reproducibility of extraction methods.

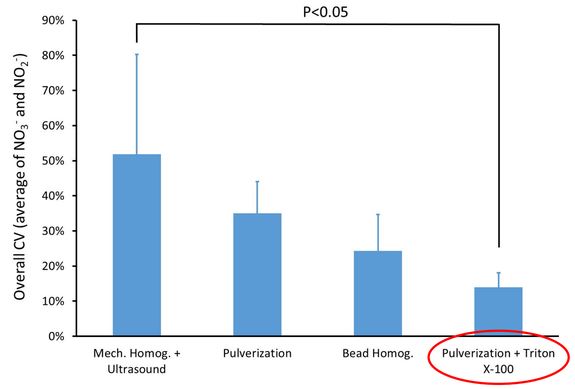
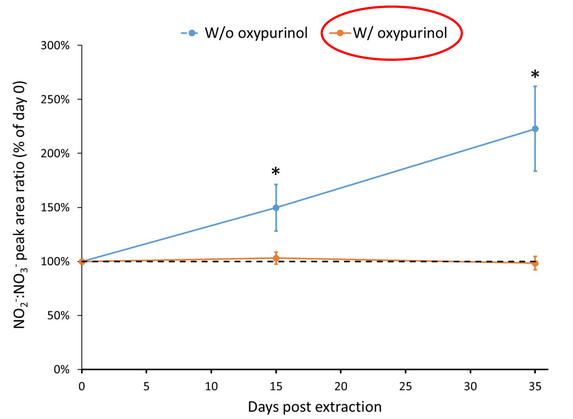


Figure 8. Effect of oxypurinol during storage at -80°C.



Conclusions

We have developed an HPLC method capable of measuring NO_3^- and NO_2^- in muscle samples as small as 5-10 mg. The theoretical limit is even lower, i.e., 1 mg for both NO_3^- and NO_2^- or 5 ng for NO_3^- alone. This method should prove highly useful in investigating the role of skeletal muscle NO_3^-/NO_2^-/NO metabolism in both healthy and diseased subject populations, in response to exercise and dietary interventions, etc.

References

1. Piknova et al. Skeletal muscle as an endogenous nitrate reservoir. *Nitric Oxide* 2015; 47:10-16.
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4. Nyakayiru et al. Sodium nitrate ingestion increases skeletal muscle nitrate content in humans. *J Appl Physiol* 2017; 123:637-644.

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