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# Urinary myoglobin quantification by high-performance liquid chromatography: An alternative measurement for exercise-induced muscle damage

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ABSTRACT

This study investigated a means of quantifying urinary myoglobin using a novel reverse-phase high-performance liquid chromatography (RP–HPLC) method that is an alternative measure of exercise-induced muscle damage. It also investigated the effect of storage and alkalization on urinary myoglobin stability issues. An RP–HPLC method was validated by precision and repeatability experiments. Myoglobin stability was determined through spiked urine samples stored at various temperatures over an 8-week period using alkalization and dilution in a pH 7.0 buffer. The method was validated with urine collected from mixed martial arts fighters during a competition and training session. The method produced linearity from 5 to 1000 μg/ml ($R^2 = 0.997$), intra- and inter-assay coefficients of variation from 0.32 to 2.94%, and a lower detection limit of 0.2 μg/ml in the final dilution and 2 μg/ml in the original urine sample. Recovery ranged from 96.4 to 102.5%, myoglobin remained stable at 4 °C when diluted in a pH 7.0 buffer after 20 h, and a significant increase ($P < 0.01$) and an identifiable peak were observed following a mixed martial arts contest and training session. Storage length and conditions had significant effects ($P < 0.05$) on stability. The method's simplicity and noninvasive nature means it can be used as an alternative muscle damage assay following exercise and trauma.



Myoglobin, the hemoprotein that facilitates oxygen storage for sustained oxidative phosphorylation in muscle fibers, is a key marker of muscle damage that has notably been employed to assess the severity of injury and monitor the recovery of individuals suffering from rhabdomyolysis [1], myocardial infarction [2], accidental trauma [3], and exercise [4,5]. Muscle damage quantification is also pertinent to the diagnosis of exercise-induced physiological stress for the management of athlete recovery and performance. To this end, myoglobin has been routinely measured in several exercise studies ranging from rugby union [6,7] to ultra-endurance exercise [8].

The methodologies employed for myoglobin measurement include sensitive and expensive immunoassays, dipsticks, histochemical staining, radioimmunoassays, and relatively inexpensive liquid chromatograph assays [6,9,10]. Enzyme-linked immunosorbent assays (ELISAs) are capable of quantifying myoglobin concentrations through their rapid and sensitive processing time. However, de Waard and van't Sant [11] highlighted ELISAs' instability, inaccuracy, and limited detection range (~1 μg/ml), suggesting that all immunoassay-based myoglobin assays are unsuitable. Similarly, urinary dipsticks are unable to differentiate clearly between myoglobin and hemoglobin and do not offer a quantification method [12]. When concentrations range from 3.2 to 3000 mg/L following serious trauma, myocardial infarction, and professional rugby [2,4,13], repeated handling and the inability to specifically quantify muscle damage severity make immunoassay options rather impractical. Likewise, compounding effects such as pH, temperature, freezing, and unidentified urinary compounds smaller than 10 kDa routinely affect stability and the ability to accurately quantify myoglobin [14].

The need for a noninvasive, reliable, repeatable, simple, and cost-effective alternative assay for the quantification of muscle

*Abbreviations:* ELISA, enzyme-linked immunosorbent assay; RP–HPLC, reverse-phase high-performance liquid chromatography; UV, ultraviolet.

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damage following physical exercise is essential. Creatine kinase and lactate dehydrogenase are routinely measured in exercise research through plasma analysis [15,16] but can be severely limited by its delayed response to the muscle-damaging exercise [17] and its invasive approach, providing robust reasoning for the requirement of an assay that can provide an immediate noninvasive quantification of muscle damage.

Furthermore, several size exclusion, anion exchange, and reverse-phase high-performance liquid chromatography (RP–HPLC) techniques have been used for protein and myoglobin determination [2,18,19]; however, they are somewhat outdated, coelute proteins of similar size, are unvalidated in urine, or require more than one preparative process. RP–HPLC methods commonly use water and an organic solvent for separation and identification that have shown a recovery of approximately 100% following addition to an extract [20]. We have adopted this methodology to develop, validate, and report a similarly reliable RP–HPLC method that quantifies myoglobin in urine by simple ultraviolet (UV) detection that negates the use of invasive procedures.

The aims of this research were to develop a simple and noninvasive RP–HPLC method that can reliably quantify urinary myoglobin following exercise-induced muscle damage as an alternative to commonly used invasive approaches and to investigate the effect of alkalization and sample dilution on urinary myoglobin stability for sample preparation optimization.

## Materials and methods

All solutions and reagents were prepared with water purified using the NANOpure ultrapure water system from Barnstead/Thermolyne (Dubuque, IA, USA). Chemicals and reagents were supplied from Sigma Chemical (St. Louis, MO, USA), Abcam (Melbourne, Australia), and BDH Chemicals New Zealand (Auckland, New Zealand).

### *Precision studies*

Spectral analyses of commercially supplied pure human (Abcam, ab96036) and equine (Sigma–Aldrich, M0630) skeletal myoglobin were conducted to identify the optimal absorbance level of myoglobin, whereas spectral analysis at 210, 280, and 400 nm were conducted by RP–HPLC on spiked myoglobin urine samples to identify any interference from other urinary compounds. Intra-assay precision was evaluated using 20 replicates of a single urine sample in a single analytical run spiked with 10, 100, and 1000 μg/ml myoglobin standard. Inter-assay precision was evaluated using 20 replicates of a single urine sample on 4 consecutive days for each of the three concentrations while a calibration curve was established using myoglobin standards ranging from 5 to 1000 μg/ml for both human and equine skeletal myoglobin. The lower detection limit for the assay was established using spiked urine samples where the peak was three times greater than the baseline noise, as described previously [19].

### *Recovery and stability*

The recovery and stability of the myoglobin was investigated by spiking a urine sample with known concentrations of human myoglobin standard (10–1000 μg/ml). Standards ranging from 10 to 1000 μg/ml were added to urine samples before being diluted 1:10 with 10 mM ammonium acetate (pH 7.0) and analyzed every 4 h for a 28-h period at 4 °C. This was to replicate standard urine collection protocols. This time period was specifically chosen due to the length of each assay (32 min) while accounting for as many as 40 samples in any one analytical run.

Because myoglobin is notoriously unstable in urine when measured by ELISA [14], the stability of myoglobin in a urine sample over an extended period of time was examined to determine percentage recovery. To a urine sample, 10, 100, and 1000 μg/ml human myoglobin standard was added and diluted 1:10 with 10 mM ammonium acetate (pH 7.0) before being either (i) frozen at −80 °C, (ii) frozen at −20 °C, or (iii) refrigerated at 4 °C for 1, 2, 4, and 8 weeks before thawing and analysis to determine stability and degradation kinetics. All experimental research was conducted in triplicate.

### *Clinical subjects and experimental design*

This analysis methodology was validated from urine collected from 14 mixed martial artists (178.4 ± 8.4 cm, 84.3 ± 12.9 kg, 26.6 ± 6.2 years), following a simulated contest ($n = 10$) and contest preparation training session ($n = 13$), who were informed of the risks involved in the study before their written consent was obtained. The simulated contest consisted of three rounds of 5 min with 60 s between rounds, whereas the training session consisted of individual fighting for three rounds of 5 min followed by sparring, wrestling, and fitness for a total session time of approximately 75–90 min. Samples were collected using a 70-ml collection pot midstream before, post, and 1 and 2 h post each event. At the time of collection, all samples were pH corrected and diluted with 10 mM ammonium acetate (pH 7.0) (1 ml urine:4 ml buffer) and placed on ice immediately at location. They were transported back

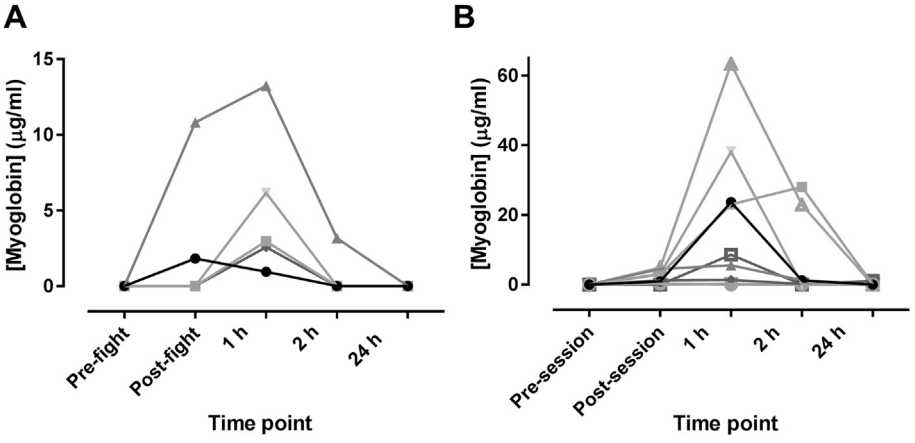


**Fig. 1.** Individual subjects' urinary myoglobin concentration–time course changes for the MMA contest (A) and training session (B).