Biochemical analysis of myoglobinuria associated with rhabdomyolysis

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Summary

The destruction of skeletal muscle in a condition known as rhabdomyolysis results in the release into the blood stream of large quantities of the 17 kDa myoglobin that freely filtrates through the glomeruli, often overwhelming the capacity of the proximal tubule for reabsorption. As a result, identification of myoglobin in the urine becomes an essential tool to fully complement other biochemical parameters in the disease diagnosis. Using a combination of protein electrophoresis in agarose gels and immunofixation with specific antibodies, we provide direct evidence for the presence of intact myoglobin in the urine of a patient with acute kidney injury due to rhabdomyolysis triggered by undesirable side effects of specific medications. The electrophoretic/immunochemical data was further corroborated by amino acid sequence, Western blot, and mass spectrometry analysis. The simple combination of electrophoresis and immunofixation protocols provides a flexible approach that can be extended to the identification of the various proteins known to be involved in overflow proteinuria.

Key words: protein electrophoresis * overflow proteinuria * myoglobinuria* rhabdomyolysis

Resumen

La destrucción del músculo esquelético en la condición patológica conocida como rabdomiolisis resulta en la liberación al torrente sanguíneo de elevadas concentraciones de la proteína mioglobina de 17 kDa, la cual filtra libremente a través del glomérulo sobrepasando frecuentemente la capacidad de reabsorción del túbulo proximal. Por lo tanto, la identificación de mioglobina en orina es una herramienta esencial que complementa otros parámetros bioquímicos en el diagnóstico de la enfermedad. En el presente
Electrophoresis is a versatile technique in the clinical laboratory that is routinely utilized to analyze complex mixtures of proteins in biological fluids (e.g., serum, CSF, urine) based on the differential charge exhibited by their different components at a given pH. In general terms, the electrophoretic pattern of plasma proteins is delineated by the differential distribution of 14 major components (1). Under normal conditions, this electrophoretic profile remains relatively constant whereas changes in the number of components and/or their concentration are either associated with specific pathogenic processes or simply reflect genetic differences without pathologic connotations. It is perhaps in cases in which the presence of extra-components is suspected that this technique is most frequently utilized, generally in combination with additional identification approaches requiring the use of specific antibodies (e.g., immunoelectrophoresis, immunofixation, immunoblot, ELISA). The classic example is the detection and identification in serum and/or urine of monoclonal components—intact or fragmented immunoglobulins and/or free light chains—associated with B cell dyscrasias (2).

The methodology becomes more relevant in situations in which the homogeneous spikes are not reactive with antibodies against immunoglobulin heavy or light chains. It is precisely in those cases where the clinical data plays a key role in the selection of the proper panel of antibodies, which may include those recognizing tumor-associated proteins including α-fetoprotein, lysozyme or β2-microglobulin, or molecules involved in tissue damage, like amylase, hemoglobin or myoglobin. The present report describes the biochemical approach to the identification of myoglobin in the urine of a reversible case of rhabdomyolysis triggered by undesirable secondary effects of lipid-lowering drugs.

**Materials and Methods**

**PATIENT INFORMATION**

A 65-year-old male patient with a history of hypertension and paroxysmal atrial fibrillation—medicated with anti-hypertensives and Coumadin—attended a routine physical examination and, after general laboratory review, was started on a regimen of statins in combination with fibrates for hypercholesterolemia and triglyceridemia. Three months later he returned to the hospital with severe symptoms of bilateral lower extremity weakness. The initial concern was spinal cord compression due to paraspinal hematoma as a result of the chronic anti-coagulant treatment. However, on admission labs, the patient showed high levels of serum transaminases,
BUN and creatinine, proteinuria and dark tea-colored urine. Initial values of CK were highly elevated (74,153 U/L) and continued to rise, peaking three days later (139,980 U/L); serum, CSF and urinary specimens were collected for electrophoresis.

**SERUM ELECTROPHORESIS AND IMMUNOFIXATION**

Semi-automated agarose electrophoresis and immunofixation were performed with the Hydrazys electrophoresis system (Sebia, Inc., Norcross, USA) according to the manufacturer’s instructions (3). For protein electrophoresis, 10 μL of either serum, 6-fold concentrated CSF (original protein content: 30 mg/dL) or 30-fold concentrated urine (original protein content: 900 mg/24 h; urine volume in 24 h: 600 mL) were manually applied to the sample template and allowed to diffuse for 5 min. Electrophoresis (pH= 8.6, 20 W, 20 °C, 7 min), drying (65 °C, 10 min), staining with amido black (4 min), destaining, and final drying (75 °C, 8 min) steps were performed automatically by the system. The gels were finally scanned with the Hyrys (Sebia, Inc., Norcross, USA) densitometer.

For immunofixation, six comparable aliquots of serum, concentrated CSF, and concentrated urine samples were separated by electrophoresis (pH= 8.6, 20W, 20 °C, 9 min) and subjected to individual immunoreactions with monospecific antisera to the human immunoglobulin light chains (κ, λ) and the major heavy (γ, α, μ) chains. This process was followed by the automatic steps of staining with violet blue, destaining, and drying. In a parallel experiment, concentrated urine was subjected to the immunofixation protocol using polyclonal anti-myoglobin antibody (Dako) using identical experimental conditions to those described above.

**AMINO ACID SEQUENCE ANALYSIS**

N-terminal sequence analysis of the homogeneous urinary band was carried out via automatic Edman degradation on a 494 Procise Protein Sequencer (Applied Biosystems, Foster City, USA). A 10 μL sample of the concentrated urine was separated on agarose gel using the same conditions as above, transferred by contact blot to polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, USA) and stained with 0.1% Coomassie Blue R-250 in 50% methanol, as described (4). The homogeneous band in the β-region was excised from the membrane and subjected to N-terminal sequencing.

**WESTERN BLOT ANALYSIS**

Five microliters sample of the concentrated urine were separated by 12% SDS-PAGE and electro-transferred to Immobilon-P membrane (1h, 400 mA) using 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer, pH=11.0, containing 10% (v/v) methanol. After transference, the membrane was blocked for 1h at 37 °C with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 and allowed to react with polyclonal anti-myoglobin antibody (Dako, Carpenteria, CA; 1:1000) followed by horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Biosource/Invitrogen; 1:5000). Fluorograms were developed by enhanced chemiluminescence (ECL) with ECL Western blotting detection reagent (GE Healthcare) and exposed to Hyperfilm ECL (GE Healthcare), as described (4).

**MASS SPECTROMETRY ANALYSIS**

The molecular mass of the homogeneous urinary band was assessed via matrix-assisted laser desorption time of flight ionization mass spectrometry (MALDI-ToF-MS) at the New York University Protein Analysis Facility. A 30-fold concentrated urine sample (1 μL) was mixed with 1μl of 10 mg/mL α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, St. Louis, MO) in 50% acetonitrile and 0.1% trifluoroacetic acid, and 1 μL of the mixture spotted on an aluminum plate, air-dried, and analyzed on a Micromass ToFSpec-2E mass spectrometer (Bruker Daltonics, Bremen, Germany) in linear mode using standard instrument settings, as previously described (5). Internal and external calibration were carried out using trypsinogen (average mass = 23981.9) and horse myoglobin (average mass = 16952.5).

**Results**

The electrophoretic analysis of the serum, urine, and CSF proteins of the patient being studied are illustrated in Figure 1. Total protein values for serum (7.5 g/dL) and CSF (30 mg/L) were within normal levels and the electrophoretic patterns were unremarkable. This was not the case of the urine specimen; a moderate proteinuria (0.9 g / 600 mL) was recorded in tea-colored 24 h urine and a homogeneous component that accounted for 52% of the urinary protein was clearly visible in the β-region after electrophoresis. Standard immunofixation protocols designed to identify the presence of monoclonal immunoglobulins and/or free light chains in biological samples rendered negative results in all three biological fluids, leaving the homogeneous component present in the urine still unidentified.

The biochemical identification of the unknown urinary homogeneous component was carried out through N-terminal amino acid sequence analysis. For this purpose, concentrated urine was separated by electrophoresis in agarose gel, transferred to PVDF, stained, and the band of interest—illustrated in Figure 2—excised from the membrane, and subjected to automatic Edman degradation. The first 10 cycles rendered the

sequence GLSDGEWQLV which, upon search at the data bank of the Swiss Institute of Informatics (http://expasy.org), revealed 100% identity to the 10 N-terminal amino acids of human myoglobin. Immunofixation analysis using polyclonal anti-myoglobin antibody specifically recognized the homogenous component (Figure 2, inset), further confirming its identity.

A brief review of the literature indicated that myoglobinuria was frequently associated with the presence of proteolytic fragments of myoglobin rather than the intact molecule, likely due to the proteolytic action of different enzymes found in the urine. In order to verify the protein’s molecular integrity, a concentrated urine sample was separated by SDS-PAGE, transferred to a PVDF membrane, and subjected to Western blot analysis probed with anti-myoglobin. As illustrated in Figure 3A, no heterogeneity was detected; only a single band with a molecular mass in the range of 17 kDa was highlighted.
by the antibody, a mass compatible with the intact molecule. Figure 3B shows the MALDI-TOF mass spectrometry analysis of the homogeneous component, revealing a molecular mass of 17053.10 Da (M+H) in agreement with the theoretical mass of 17052.63 (M+H), and confirming that the urinary myoglobin molecule was not degraded.

Discussion

The term proteinuria relates to the presence of proteins in the urine that exceeds the amount of 150 mg in 24h (6). Passage of molecules across the glomerular barrier is a function of size, charge and configuration. Molecules with an effective radius < 2.0 nm readily filter while those with higher size are partly or completely retained. As a consequence, proteins with a relative molecular mass >40 kDa are almost completely retained whereas smaller components easily enter the glomerular filtrate. However, size is not the only element restricting glomerular filtration. Charge plays also a critical role and negatively charged components of the glomerular basement membrane favor the retention of anionic molecules.

Most of the proteins in the glomerular filtrate are reabsorbed and metabolized at the tubular level. Under certain pathologic circumstances, increased synthesis or release to the circulation of specific low molecular

Figure 2. Biochemical analysis of the urinary homogeneous component. The main panel illustrates the PVDF membrane after contact-blot transfer of the electrophoresed urine sample. Arrow highlights the band that was excised from the membrane and subjected to N-terminal sequence analysis yielding the sequence GLSDGEWQLV which corresponded to the intact N-terminus of myoglobin. Inset depicts the immunofixation analysis in Hydrasys electrophoresis system illustrating the electrophoretic pattern of the specimen in the left lane and the immunoreactivity with anti-myoglobin in the right lane.

Figure 3. Electrophoresis in SDS-polyacrylamide gels, Western blot, and mass spectrometry analysis. (A) SDS-PAGE separation of the urine sample (lane 1) illustrating the molecular weight of the predominant homogeneous urinary band and the immunoreactivity of the major component with anti-myoglobin antibodies (lane 2). (B) Mass spectrometry analysis of the urine sample highlighting the peak of a molecular mass of 17053.1 Da, corresponding to the molecular mass of the intact molecule.
mass proteins results in an elevation of their plasma concentration, saturating the tubular re-absorption mechanism and translating in their appearance in the urine, a process known as “overflow proteinuria” (7). Table I summarizes the most frequent pathologic conditions and their corresponding protein components associated with overload proteinuria. Within this group, the most common finding is related to the presence of monoclonal immunoglobulins, in particular free light chains (Bence Jones proteins), typically associated with overproduction in cases of lymphoproliferative diseases and multiple myeloma. Due to the low molecular mass of these light chains (25 kDa for the intact molecule and variable smaller size when proteolytically degraded) they massively filterate through the glomeruli, surpassing the capability of the tubular reabsorption and making them clearly visible in the urine. Although less frequent among the molecules associated with overload proteinurias (8), lysozyme is found in the urine in cases of acute granulocytic and monoblastic leukemias since this 14 kDa protein is highly concentrated in granulocytes and monocytes. In these leukemias there is an abnormal elevation of lysozyme in plasma that overwhelms the re-absorption machinery resulting in its presence in the urine (9-11). Another molecule that may be identified in overload proteinurias is β2-microglobulin (12), typically elevated in lymphoproliferative processes in which high serum levels of the protein (above 6 mg/L) are usually indicative of poor disease prognosis (13). It should be noted that lysozyme and β2-microglobulin have similar molecular mass, comparable serum concentration, and are both reabsorbed at the tubular level; therefore, their presence in the urine may not only be indicative of overload proteinuria –due to increased serum concentration with preserved renal function– but may reflect an alteration of the renal tubular re-absorption, as in the case of tubular proteinurias (14). In this sense, β2-microglobulin is a well known indicator of renal tubular dysfunction, widely used in the clinical setup (15). Among other molecules associated with overload proteinurias it is pertinent to cite amylase and hemoglobin (12). Amylase, a 45 kDa pancreatic protease, can be transiently found in the urine of patients with acute pancreatitis (12) whereas the presence of hemoglobin in urine is primarily associated with hemolytic anemia of different origins –including autoimmune conditions, blood transfusion reactions, and paroxysmic hemoglobinuria– as well as with multi-trauma situations in which the destruction of red blood cells occurs massively and in a short period of time transiently overwhelming the tubular reabsorption mechanisms. Finally, the presence of myoglobin in the urine –as in the case of the present report– is another example of overload proteinuria. Visible myoglobinuria occurs when urinary myoglobin exceeds 250 mg/L, which corresponds to the destruction of more than 100 g of muscle tissue (16). Both, myoglobinuria and hemoglobinuria are quite common in hospitalized patients and occur in association with a variety of diseases. Myoglobinuria can be inferred if urinary dipstick testing shows a positive result for blood in the absence of red cells in the sediment. This false positive result for blood occurs because the dipstick test—with a sensitivity of 80% for the detection of myoglobinuria—is unable to distinguish between myoglobin and hemoglobin (17).

In normal subjects, urinary myoglobin is usually below the detection limits of most methods and is probably <0.4 mg/L. Under conditions of severe exercise, myoglobin concentration in serum can increase up to 40-fold with a concomitant high increase in the urinary concentration without the risk of acute renal failure. Thus, it can be inferred that values <15 mg/L can be tolerated by the kidney without risks. The symptoms and history of myoglobinuria are obvious in certain clinical situations such as in crush injuries or severe burns. In about one-fourth of the cases, particularly in non-traumatic rhabdomyolysis, the symptoms are vague and biochemical analysis is necessary for diagnosis. Milder forms of rhabdomyolysis may not cause any muscle symptoms while more severe types exhibit intense myalgia, tenderness, weakness and swelling of the affected muscles (18). If the swelling is very rapid, as it may happen when someone is released from under a collapsed building, the movement of fluid into the blood stream may cause hypotension and shock. Other symptoms are non specific and result either from the consequences of muscle breakdown or from the condition that originated it (18-20). Release of muscle components into the bloodstream causes disturbances of electrolytes, which can lead to nausea, vomiting, confusion and even coma in the most severe cases. Non-traumatic myoglobinuria with acute renal failure is a relatively common disease in patients with alcohol overdose, a history of drug addiction, or experiencing side effects to specific medications. In adults, drug-induced muscle damage account for approximately half of all rhabdomyolysis cases. In

Table 1. Proteins commonly identified in overflow proteinurias and corresponding clinical entities

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<tr>
<th>OVERFLOW PROTEINURIAS</th>
<th>Monoclonal Gammapathies</th>
<th>Immunoglobulin Heavy and/or Light Chains</th>
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<tr>
<td>Acute Granulocytic Leukemias</td>
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<td>Lysozyme</td>
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<td>Lymphoproliferative Disorders</td>
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<td>β2-microglobulin</td>
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<td>Acute Pancreatitis</td>
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<td>Hemolysis (after Haptoglobin saturation)</td>
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<td>Rhabdomyolysis</td>
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addition to alcohol abuse and consumption of illegal substances including cocaine, amphetamines, Ecstasy and LSD, there are close to 200 medications that have been implicated in the development of rhabdomyolysis (21-23). Antipsychotic drugs, corticosteroids and HIV medications have all been reported to cause rhabdomyolysis but undoubtedly one of the most relevant are statins, used to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase, which plays a central role in the production of cholesterol in the liver (23). Although the risk of serious myopathy from statins is less than 0.1%, the wide use of these drugs makes screening patients for muscle damage a very important follow up criteria. Statins differ among themselves in several ways including in their ability to reduce cholesterol with current data indicating that atorvastatin—the best selling of the group—and rosuvastatin are the most potent, and fluvastatin is the least potent (24). Statins also differ in how strongly they interact with other drugs, an important element in patients receiving combined therapeutic regimes. Most critical for the current studies, statins differ also in the frequency with which they cause severe muscle damage. A recent retrospective review of records from over 250,000 patients revealed that the incidence of rhabdomyolysis was 0.44 per 10,000 patients treated with statins other than cerivastatin (25) (26). However, the risk was over tenfold greater if cerivastatin was used, which resulted in the drug's withdrawal from the market by its manufacturer in 2001. The risk of rhabdomyolysis was also notably enhanced when standard statins (atorvastatin, fluvastatin, lovastatin, pravastatin, simvastatin) were combined with fibrate (fenofibrate or gemfibrozil) treatment, as in the case reported herein.

Rhabdomyolysis accounts for 5% to 7% of all cases with acute renal failure and myoglobinuria is, therefore, a frequent finding in patients requiring dialysis (27). It is unclear why heme pigmenturia is associated with acute renal tubular necrosis. The difficulty with which acute renal failure is induced with pure myoglobin or hemoglobin solutions in experimental animals, as well as the absence of renal pathology in about half the patients with muscle phosphorylase deficiency that show spontaneous myoglobinuria suggests that coincident factors may be required to enhance the toxicity of hemoproteins to the kidney, among them dehydration and hypotension (27-28).

In addition to the presence of myoglobin in urine, rhabdomyolysis is usually accompanied by other biochemical parameters, including greater than 40-fold increase of serum myoglobin and elevated levels of several serum enzymes such as CK (>40-fold increase) as well as aspartate aminotransferase (>4-fold increase) and LDH (>2-fold increase) (27). Urine myoglobin is the first test analyte to increase; it subsides rapidly within the first few days, reflecting its small molecular mass and its half-life of about 2-3 hours. CK increases within a few hours after muscle damage but remains elevated a few days longer than myoglobin. It is important to remember that urine is a hostile environment for proteins in general. It contains several proteolytic enzymes and various salts that can denature or hydrolyze proteins. As it occurs with other proteins, myoglobin most frequently brakes down in urine although it is stable for a few days in the fridge. Thus, the sooner the sample is processed, better chances for the successful identification of the components that are inducing the overload proteinuria.

The case illustrated herein presented with a combination of renal failure, liver compromise, and muscle weakness that oriented the diagnosis towards rhabdomyolysis, which was confirmed by the biochemical identification of myoglobin in the urine. In this particular situation, rhabdomyolysis was linked to the undesirable side effect of statins. The medication was halted and after approximately 2 weeks of hemodialysis for the renal failure the patient fully recovered his renal function and his enzymatic profile normalized. Due to remaining severe bilateral lower extremity weakness that precluded him to ambulate, he was transferred to a sub-acute rehabilitation facility for further treatment. In summary, this report highlights the relevance of the clinical laboratory as a key player in diagnosis emphasizing its role in cases of overflow proteinurias. Although homogeneous components appearing in protein electrophoresis studies most frequently consist of intact or truncated monoclonal immunoglobulins, additional unrelated protein spikes should be taken into consideration in relation to different pathological entities. As illustrated herein, simple modifications of routine methodologies available to the clinical laboratory allows the identification of uncommon proteins in biological fluids.

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