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Myoglobinuria detection by capillary electrophoresis

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Abstract

Capillary electrophoresis was used in this study to separate urinary myoglobin from hemoglobin based on its electrophoretic mobility. Urine was applied directly without any treatment. The separation was accomplished in less than 7 min. Myoglobin extracted from human muscle tissues was separated, in a borate buffer 150 m*M*, pH 8.7 containing 0.5% polyethyleneglycol at 6 kV, into two peaks (MI and MII) which were also resolved far from hemoglobin. Upon standing at room temperature, MII converted into MI. Horse myoglobin eluted close to MI.

The addition of polyethyleneglycol to the buffer enhanced the separation and increased the peak height of myoglobin. Optimum conditions for the separation are discussed. The method is suitable for routine clinical analysis because of its simplicity and speed.

1. Introduction

Myoglobinuria, the presence of myoglobin in the urine, occurs in patients with different disorders, such as trauma infections and burns, reflecting muscle tissue breakdown [1]. It is important to recognize this condition as soon as possible to start the patient on the appropriate therapy to prevent acute renal failure [2–4].

Hemoglobin is the main pigmented protein encountered in urine which can interfere with myoglobin detection. The separation of these two proteins may appear simple; however, in practice it is very difficult [4]. Since myoglobin tends to denature rapidly in urine, within a few hours, rapid methods for its detection are needed.

Several techniques have been advocated for the detection of myoglobin such as agarose electrophoresis, salt precipitation, immunoassays, and spectrophotometry as summarized in Ref. [4]. None of these methods is very practical for routine clinical use because of either a lack of sensitivity or the need for time-consuming steps [4].

Here we describe the use of a simple capillary electrophoresis (CE) method to separate and quantitate myoglobin from hemoglobin. The method is rapid, less than 7 min, using urine directly without sample extraction, concentration or staining.

2. Experimental

2.1. Instrument

A 30 cm × 50 μm I.D. untreated silica capillary was used in a Model 2000 CE instrument (Beckman Instruments, Palo Alto, CA, USA) equipped with a 405-nm filter. The voltage was set at 6 kV. The electrophoresis buffer was borate 150 m*M* pH 8.2 with 0.5% polyethyleneglycol (PEG) 8000. Samples were intro-

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duced by pressure injection for 6 s. The capillary was washed between injections with 7 mmol/l phosphoric acid for 40 s followed by the electrophoresis buffer for 60 s.

2.2. Chemicals

Horse myoglobin was obtained from Sigma Chemicals (St. Louis, MO, USA). Polyethyleneglycol 8000 was purchased from Fisher Scientific Company (Fairlawn, NJ, USA).

2.3. Tissue extraction

Human skeletal muscle tissue samples from autopsy materials (200 mg) were homogenized in 1.0 ml water and centrifuged for 1 min at 14 000 *g* in a Beckman microfuge. The supernatant was used for analysis.

2.4. Internal standard

Washed red blood cells were hemolyzed in water to give a solution of hemoglobin (Hb) of about 200 mg/l. Hemolysate, 25 μl, was mixed with 25 μl urine and the Hb peak was used as an internal standard.

2.5. Urine samples

Urine samples were refrigerated and assayed within 4 h of receiving. The urine samples were either diluted with an equal volume of water or water containing the internal standard.

3. Results and discussion

Using a borate buffer, the electrophoretic separation of human myoglobin freshly extracted from muscle tissue reveals two peaks: a large one (MII) and a smaller one (MI) with a faster mobility (Fig. 1). However, after the sample stands at room temperature for a few hours, the smaller peak increases in height and finally becomes the major one. This indicates that myoglobin undergoes a charge change, either losing a negative charge or exposing a positive one, causing the molecule to become more cationic and thus migrating faster. We have seen the same phenomena previously on an anionic HPLC column [5]. The two peaks were designated as MI and MII [5]. Since this separation was not observed on a Spherogel 2000 column, the peak transformation was thought to reflect a charge change rather than a peptide breakdown [5]. Urine from patients with myoglobinuria, as expected, has mainly the MI peak (Fig. 2) [5]. This can be confirmed by the addition of fresh-tissue-homogenate myoglobin to the urine of patients with myoglobinuria.

3.1. Effect of voltage

Fig. 3 illustrates the effect of voltage on the peak height and electrophoretic mobility. As the voltage increases from 3.5 to 13.5 kV the migration time decreases; however, the peak height decreases too, probably due to denaturation of the two proteins.

3.2. Ionic strength

Fig. 4 illustrates the effect of ionic strength on the separation and migration of myoglobin. As the ionic strength increases from 25 to 150 m*M*, the rate of migration increases while the peaks for both myoglobin and hemoglobin sharpen, i.e. increase in plate number. A further increase in the ionic strength to 200 m*M* sharpens the myoglobin peak more but the hemoglobin peak becomes broader and decreases in height. It seems that at this concentration hemoglobin starts to denature. It is known that hemoglobin precipitates before myoglobin in highly concentrated solutions of ammonium sulfate [6]. For this reason a 150 m*M* borate buffer was chosen for the optimum ionic strength in order to detect the two proteins.

3.3. Effect of pH

The separation of MI and MII at pH 8.2 is slightly better than at pH 8.7, especially when using a 200 m*M* borate buffer. Increasing the pH further to 9.9 causes the MI and MII peaks to

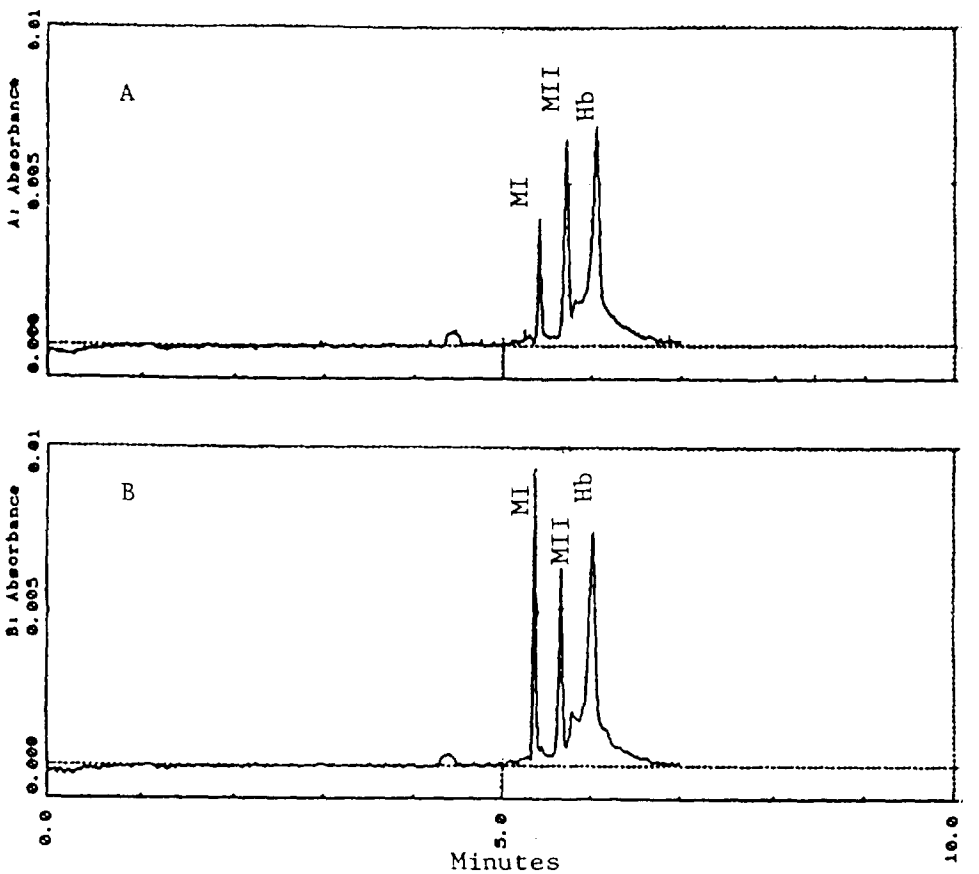

Fig. 1. Human muscle myoglobin (MI and MII). (A) Assayed immediately. (B) Assayed after 2 h at room temperature. Hemoglobin (Hb) was added as an internal standard.

co-elute. However, hemoglobin separates well from myoglobin at all three hydrogen ion concentrations used.

3.4. Polyethyleneglycol effect

The addition of 0.4% PEG to the borate buffer increases electrophoretic mobility for both myoglobin and hemoglobin (Table 1). Enhanced separation between the two proteins occurs as the ratio of hemoglobin/myoglobin mobility time increases from 1.06 to 1.11. The improved separation probably reflects a sieving effect [7] of the PEG on the hemoglobin which has a higher molecular mass of 64 000 relative to myoglobin (with a molecular mass of 17 000). The peak height especially of myoglobin also increases with the addition of PEG because of the increase in buffer viscosity and the decrease in the binding to the capillary surface. Thus for routine analysis PEG was added at a concentration of 0.5% to the borate buffer.

3.5. Standards

Horse myoglobin migrates in the presence of polyethyleneglycol close to, but slightly ahead of, MI. Since purified human myoglobin is difficult to obtain commercially, horse myoglobin can be used as a standard for quantitation.

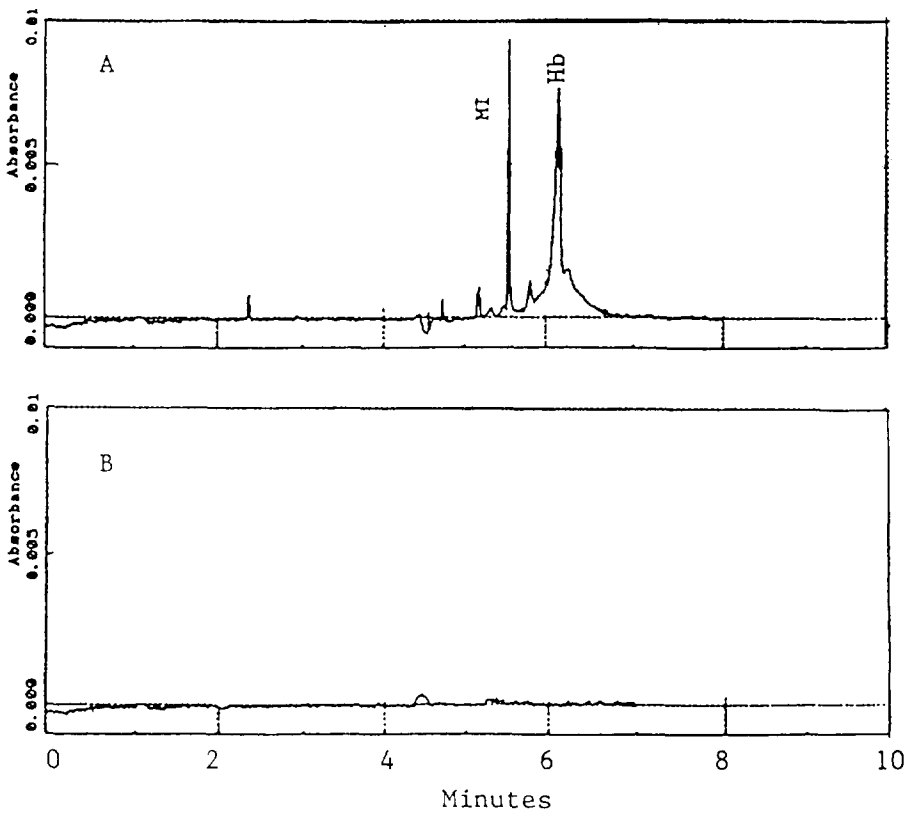

Fig. 2. (A) Urine sample from a patient with myoglobinuria (400 mg/l). (B) Urine sample from a normal subject. Hemoglobin (Hb) was added as an internal standard in (A).

3.6. Comparison with HPLC

Table 2 illustrates the comparison between the quantitation of myoglobin by an anion-exchange HPLC [5] and that by CE. Ten samples from normal individuals did not contain any myoglobin or hemoglobin. It is important to note that myoglobin is not stable. It denatures and degrades continuously even when refrigerated or frozen. Some samples probably degrade before arriving at the lab. Our attempts to solubilize myoglobin after it precipitated were not successful. Thus, the difference between the HPLC and CE values is related primarily to the difference in analysis time. In general, the CE method is much simpler than HPLC [5] mainly because of the elimination of the packed column and the pump.

3.7. Interferences

Detection at the wavelength of 405 nm offers good sensitivity and eliminates many of the interferences present in urine such as proteins, peptides and small molecules. The absorption of myoglobin at 405 nm is much stronger than that at 280 nm (Fig. 5). Hemoglobin is the peak most often detected in urine. The common hemoglobin variants A, F, S and C do not interfere with the MI peak (the main myoglobin peak in urine). The limit of detection for myoglobin is about 15 mg/l (3 × baseline noise). This level is adequate

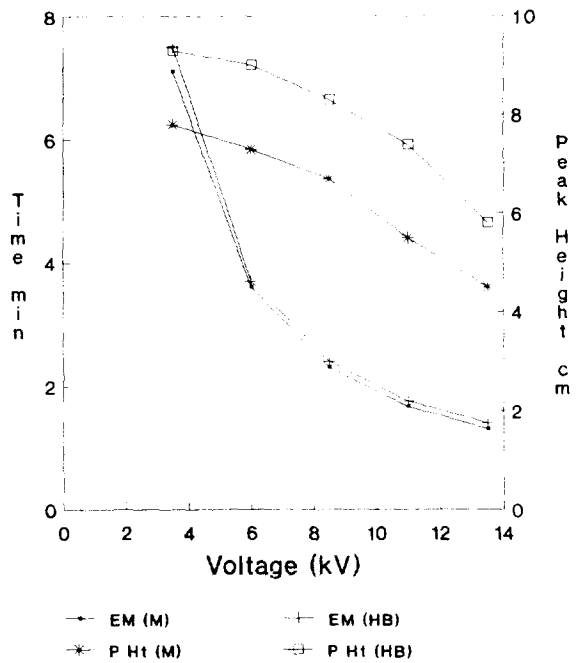

Fig. 3. Effect of the voltage on myoglobin MI (M) and hemoglobin (HB) peak heights (P Ht) and electrophoretic mobility (EM). Separation was performed with borate buffer pH 8.2, 150 m*M* without PEG.

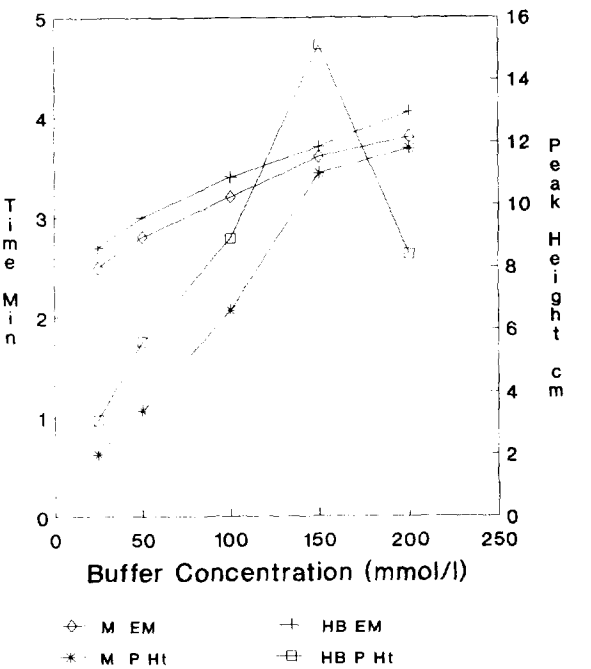

Fig. 4. Effect of the ionic strength on myoglobin MI (M) and hemoglobin (HB) peak heights (P Ht) and electrophoretic mobility (EM) at 6 kV without PEG.

for clinical detection of myoglobinuria [5]. The test is linear between 15 and 400 mg/l [myoglobin (mg/l) = 19.1 × mA − 2.14, $r = 0.999$].

3.8. Capillary life

The same capillary was used for over 100 samples without any major deterioration in the separation. The use of high ionic strength buffers with acid washing between injections [8] contributes to the good separation and long capillary life. We found some slight differences for the optimum voltage between different capillaries

Table 1
Effect of PEG on electrophoretic mobility of MI and Hb

PEG conc. (%)	Electrophoretic mobility (min)			
	0	0.4	0.8	1.2
HB	4.11	5.57	6.49	7.00
MB	4.36	6.18	7.28	7.91
Mb/HB	1.06	1.11	1.12	1.13

Table 2
Correlation of myoglobin by CE and anion-exchange HPLC method [5] ($r = 0.898$)

Sample	CE (mg/l)	HPLC (mg/l)
1	600	540
2	400	420
3	104	40
4	1230	1130
5	40	30
6	830	600
7	390	450
8	170	210
9	30	20
10	25	20
11	2440	2760
12	0	0

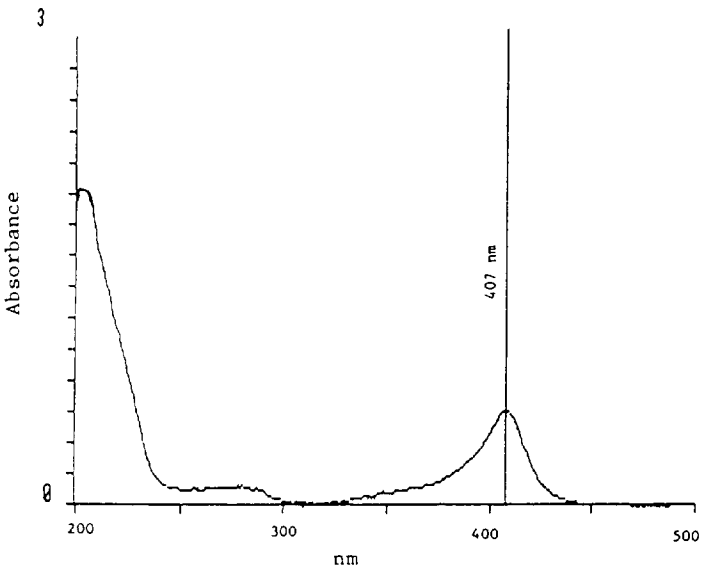

Fig. 5. The absorption spectrum of horse myoglobin (100 mg/l in borate buffer pH 8.2, 150 m*M*).

probably because of slight differences in diameter, length, etc.

3.9. Effect of the sample matrix

The electrophoretic mobility of myoglobin, as well as that of hemoglobin, changes slightly from one urine to another and also differs from that of the aqueous standards reflecting differences in the sample matrix, e.g. salt, pH and viscosity [9]. In order to overcome this problem, the analysis of the positive samples is repeated after spiking with hemoglobin (200 mg/l). Myoglobin has a distinct sharp peak different from that of hemoglobin which is easy to recognize.

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