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Determination of 3-methylhistidine and 1-methylhistidine in untreated urine samples by capillary electrophoresis

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Abstract

Capillary electrophoretic (CE) method was developed for the determination of urinary 3-methylhistidine (3MH) and 1-methylhistidine (1MH) indicating the extent of degradation of skeletal muscle proteins and thereby the state of human health. 3MH, 1MH and histidine can be separated in both acidic and alkaline media, where these amino acids form cation and anion, respectively. The effective mobility of all ionic forms was measured over a broad range of pH (1.67–11.80), which made it possible to evaluate the corresponding dissociation constants. 3MH and 1MH were determined together with creatinine in untreated urine samples with the limit of detection of 2.4 μM (0.4 mg L^{-1}) and 3.0 μM (0.5 mg L^{-1}), respectively. Determination was fast and took ca. 12 min including the column washing. Method was employed for an analysis of urine collected from healthy individuals, and from the patients hospitalized with obesity and diabetes mellitus II. This analysis has revealed differences between the healthy individuals and the patients pointing to a more extensive degradation of muscle proteins in the latter group.

Keywords: 3-Methylhistidine; Capillary electrophoresis; Mobility; Untreated urine

1. Introduction

Proteins, the most important group of biomolecules that by weight are contained mostly in muscles, undergo the continuous degradation and biosynthesis from amino acids [1,2]. Catabolism of skeletal muscle proteins, actin and myosin, can be followed, e.g. by monitoring the urinary level of 3-methylhistidine (3MH), cf. Fig. 1, which is a product of methylation on the imidazole moiety of histidine molecule occurring in the course of post-translation modifications of actin and myosin after their biosynthesis. Owing to absence of its genetic code, 3MH is not utilized in proteosynthesis, and because it is not metabolized further, it is excreted as such in the urine [3,4].

However, proteolysis is not the only source of 3MH, because significant amounts of 3MH can be taken up with the diet, in particular with the fat containing food products. Endogenic 3MH can be distinguished from the exogenic one by detecting another methylated derivative of histidine, 1-methylhistidine (1MH), cf. Fig. 1, which is not formed in humans, but it is common in other animals. The urinary level of 1MH correlates well with that of 3MH from the diet, so that their simultaneous determination can be used to follow the proteolysis of the skeletal muscle proteins [4].

The techniques that have been commonly used to analyze amino acids in biological fluids comprise ion exchange chromatography or reversed-phase liquid chromatography (LC) [5–11]. LC determinations are very sensitive but rather time consuming, because a tedious pre-treatment of a biologically sample is usually necessary prior to its injection into instrument. On the other hand, concentrations of many urinary metabolites are high enough for the capillary electrophoresis (CE) to become a convenient method of choice. A CE analysis of urine samples often requires only the sample filtration through a micro-filter coupled to a syringe followed by the sample injection into the CE capillary. While the migration

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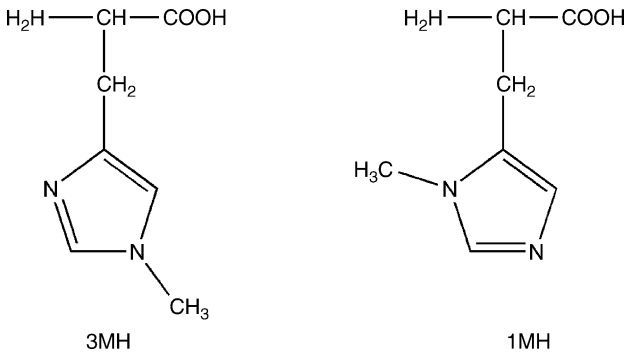

Fig. 1. Structure of 3-methylhistidine and 1-methylhistidine according to the nomenclature that is commonly found in literature. Following the IUPAC recommendation, the compound names 3MH and 1MH should be reversed.

times in CE and retention times in LC can be comparable, the total time of analysis in LC increases at least by 30 min, when the sample pre-treatment is performed [12–17].

The aim of this work was to develop a CE method for the determination of 3MH and 1MH in untreated urine. The particular attention was paid to pH of the separation electrolyte, because both 3MH and 1MH are weak electrolytes with three ionizable groups in the molecule, and can be separated as cation or anion depending on the solution pH [18,19]. Hence, the actual mobility of both methylated histidine derivatives was measured by using series of buffers to cover the pH range from 2 to 12. These results allowed us to evaluate the dissociation constants for all three dissociation steps. The CE method was then used to determine 3MH, 1MH in urine collected from healthy individuals, and from the patients hospitalized with obesity and diabetes mellitus II. Method also enabled to measure simultaneously creatinine.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade purity. NaOH, acetic acid (HAc), 2-(cyclohexylamino)ethansulfonic acid (CHES), tris(hydroxyethyl)aminoethane (TRIS), 3-methylhistidine (3MH) and creatinine (Crea) were purchased from Fluka (Buchs, Switzerland); NaH_2PO_4, Na_2HPO_4, 3-(cyclohexylamino)propansulfonic acid (CAPS), hydroxyethylcellulose (HEC), histidine (His) were purchased from Aldrich (Steinheim, Germany); H_3PO_4, dimethylsulfoxid (DMSO) and thiourea were purchased from Lachema (Brno, Czech Republic), and 1-methylhistidine (1MH) was purchased from Sigma (Steinheim, Germany). Aqueous solutions of 1 mM thiourea or 0.2% (v/v) DMSO were used alternatively as the markers of the electroosmotic flow (EOF). Milli-Q deionised water (Millipore, Bedford, USA) was used for preparation of the stock solutions of 3MH, 1MH a His (all 1 g L^{-1}) and creatinine (10 g L^{-1}).

2.2. Instrumentation and separation conditions

All the CE measurements were carried out using $HP^{3D}CE$ system (Agilent Technologies, Waldbronn, Germany) equipped with a built-in photometric diode-array detector (DAD) and controlled by the ChemStation CE software. Histidine and its methylated derivatives 3MH and 1MH exhibit a maximum absorption at 214 nm and, hence, this wavelength was used for their detection. Separation took place in a fused-silica capillary (Silica Tubing & Optical Fibers, Slovak Republic) at the controlled temperature of 25 °C. Before its first use, the capillary was conditioned by washing with 0.1 M NaOH, then with deionized water, and finally with the separation electrolyte containing the selected buffer, for 10 min each. The high voltage was then applied for 20 min so as to stabilize EOF in the subsequent measurement. Between each two CE runs, the capillary was washed with the separation electrolyte of the same pH for 1 min. All these conditioning steps were applied whenever the separation electrolyte was to change.

Electrophoretic mobility was measured in a uncoated capillary (30 cm in length, 21.5 cm in length to the DAD detector, 50 μm i.d. × 375 μm o.d.) covered with a polyimide protective layer. Solutions containing 1 mM 3MH and 1 mM 1MH were prepared freshly by dilution of their stock solutions with deionized water and analyzed apart from each other only in a mixture with the EOF marker. They were introduced with the help of the 100 mbar s hydrodynamic injection. The separation electrolytes were prepared by dissolving the corresponding acid in deionized water, to which 1 M NaOH was added to adjust pH as required. The separation NaH_2PO_4/Na_2HPO_4 buffer was prepared directly by dissolving the corresponding amounts of salts. The solution pH was measured with the help of the laboratory pH meter (pMX 3000, Wissenschafftlichen-Technischen-Werkstätten, Germany).

The CE analysis of urine samples was carried out using an uncoated capillary (65 cm length, 56.5 cm to the DAD detector, 75 μm i.d. × 375 μm o.d.) with the applied voltage of +30 kV. Before its first use, the capillary was conditioned as described above. Between each two runs, the capillary was washed with 0.1 M NaOH for 1 min, then with the deionized water for 1 min and finally with the separation buffer for 2 min. The optimized composition of the separation buffer was 0.5 M HAc, 20 mM TRIS and 0.1% HEC (w/w), pH 3.4. The urine samples were introduced with the help of the 150 mbar s hydrodynamic injection.

2.3. Urine sample preparations

Urine was collected from 10 healthy volunteers and from 20 patients hospitalized in Faculty Hospital at Královské Vinohrady in Prague 10 with obesity (10 patients) and diabetes mellitus II (10 patients). The urine samples were stored immediately after their collection at −20 °C. After defrosting, 1 mL of the sample was centrifuged for 5 min at 1500 × *g*. The supernatant was filtered using a micro-porous

Nylon filter (0.45 μm, TESSEK, Czech Republic), and injected directly into the CE instrument.

3. Results and discussion

3.1. Separation of His, 3MH and 1MH in acidic and alkaline media

His, 3MH and 1MH are commonly found in urine of healthy individuals at concentrations ranging from 1 to 10 μM L^{-1} in the order of magnitude. The carboxy, side-chain imidazole and amino group in the molecule of these α-amino acids can be characterized by the acid dissociation constants K_{a1}, K_{a2}, K_{a3}, respectively. The values $pK_{a1} = 1.7$, $pK_{a2} = 6.0$ and $pK_{a3} = 9.1$ for histidine can be found in literature [20]. The methylated derivatives 3MH and 1MH can be expected to exhibit similar values. On increasing the solution pH, the carboxy-, imidazole and amino-groups of these amino acids are in sequence deprotonized, so that their ionic form changes from the divalent cation AH_3^{2+} through the univalent cation AH_2^+ and neutral zwitterion AH to the univalent anion A^-, cf. Fig. 2. Consequently, these amino acids can be separated electrophoretically as cations or anions in acidic or alkaline media, respectively.

The effective mobility of 3MH and 1MH was measured over the broad range of pH = 1.7–11.8 by using five buffer systems based on H_3PO_4/NaOH (pH = 1.05–3.39), HAc/NaOH (pH = 2.92–5.81), NaH_2PO_4/Na_2HPO_4 (pH = 5.98–8.41), CHES/NaOH (pH = 8.16–10.86) and CAPS/NaOH (pH = 10.23–11.80). The actual pH of each buffer system was varied by 0.2–0.5 unit by adjusting the ratio of the weak acid and NaOH concentrations. Concentrations of the buffer components were chosen so as to keep the ionic strength constant and equal to 25 mmol L^{-1}, which makes it possible to use the measured mobility for the evaluation of the dissociation constants of 3MH and 1MH from the measured effective mobility [21–24]. Composition of the buffer system was calculated with the help of the simulation freeware Peakmaster [25]. Electric power of the current flowing through the capillary was kept constant and equal to 0.26 W by the properly applied separation voltage. A constant electric power should ensure maintaining the Joule heat released inside the capillary, and thereby should help to maintain the constant temperature of separation.

Several typical electropherograms are shown in Fig. 3. These results indicate that the mobility of both His and 3MH, as well as of 1MH, varies with pH. A complete separation of these three analytes is possible only at the distinct pH values. In general, when pH is lower than 7.4, all three amino acids migrate as cations. In the narrow range of pH close to 7.5, they all reach the isoelectric point, and their transport is controlled by EOF. When pH is higher than 7.6, they start to migrate as anions and they exhibit the anodic mobility.

On the other hand, the biological fluids including urine are complicated matrices containing tens to hundreds of ionizable components, which can complicate the CE analysis depending on pH of the separation electrolyte. Hence, pH is usually not a matter of the arbitrary choice. For the purpose of the CE analysis, it appears as convenient to convert organic urinary metabolites into their cationic forms, which can be easily separated from highly mobile inorganic cations that are present in urine in excess (Na^+, K^+, NH_4^+, Ca^{2+}, Mg^{2+}). An advantage of the cationic separation mode is also the possibility of the simultaneous determination of creatinine, which serves as a reference component of urine, which is excreted at a constant rate. Concentrations of various urine components can vary due to diuresis. In order to account for this effect, the results of urine analysis are usually related to the concentration of creatinine, as determined in the same sample.

The separation of 3MH, 1MH and His in urine sample was performed using several weak organic acid buffers. Formic, lactic and acetic acid were tested as components of the separation buffer, but only the latter one provided a plausible separation. For this reason acetate buffer was used further. By varying its concentration (30–1350 mM) and pH (2.9–5.8), we found the optimum concentration of 0.5 M and pH 3.4. Higher buffer concentration ensures a higher buffer capacity, which is crucial for analysis of undiluted urine. In addition, the value of the Kohlrausch regulation function for this buffer is high enough to expect that the analytes will separate in narrow zones. The adsorption of cationic forms of methylhistidines on the capillary wall was suppressed by

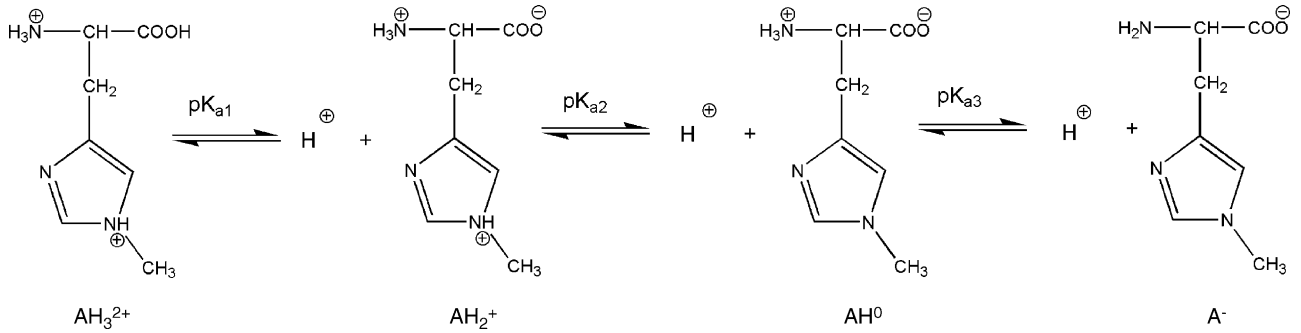

Fig. 2. Dissociation equilibria of 3-methylhistidine.

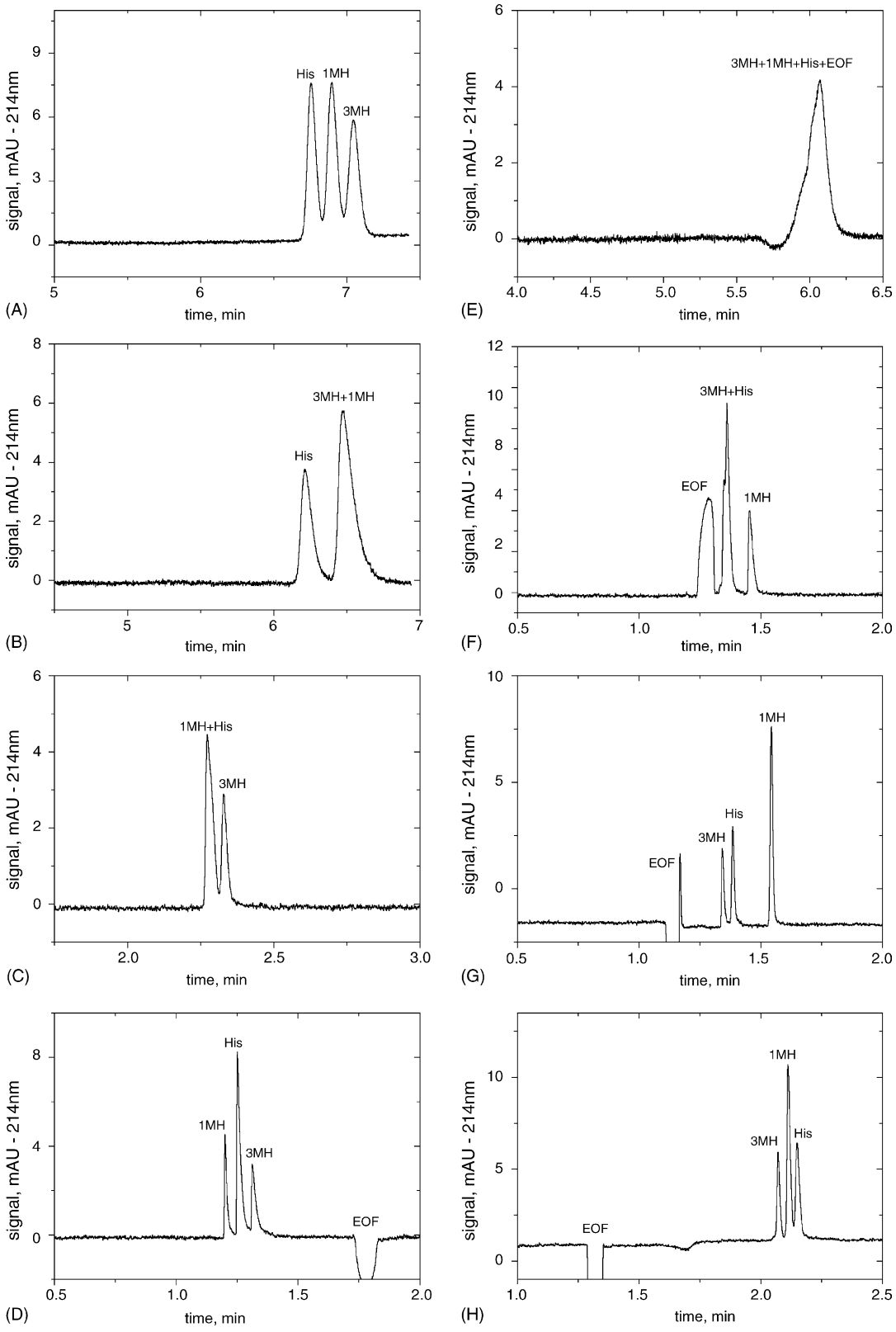

Fig. 3. Electrophoretic separation of 3MH, 1MH and His in a model aqueous sample in various buffer systems, pH and separation voltages: (A) 500 mM H_3PO_4 (pH 1.05, 4.8 kV), (B) 100 mM H_3PO_4 (pH 1.67, 5.8 kV), (C) 155 mM HAc + 25 mM NaOH (pH 3.92, 12.9 kV), (D) 29 mM HAc + 25 mM NaOH (pH 5.47, 12.7 kV), (E) 2.5 mM NaH_2PO_4 + 7.5 mM Na_2HPO_4 (pH 7.46, 14.4 kV), (F) 0.3 mM NaH_2PO_4 + 8.2 mM Na_2HPO_4 (pH 8.41, 14.3 kV), (G) 68 mM CHES + 25 mM NaOH (pH 9.27, 14.2 kV), (H) 23 mM CAPS + 25 mM NaOH (pH 11.80, 12.2 kV). Capillary: inner diameter 50 μm, effective length 21.5 cm, total length 30 cm; hydrodynamic injection 100 mbar s. Relative differences in peak height of analytes are due to various concentrations in mixtures.

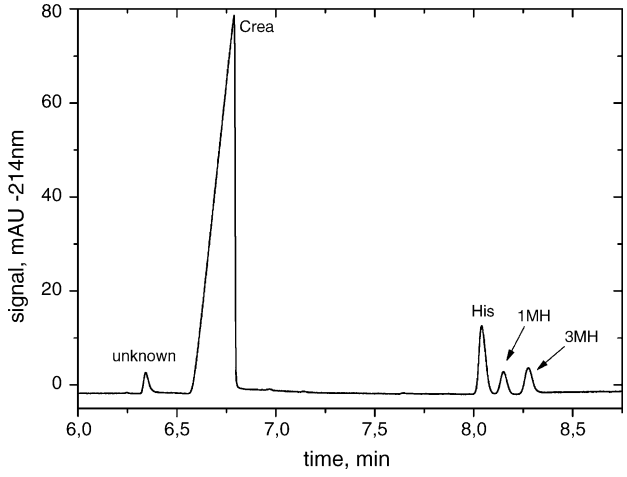

Fig. 4. Electropherogram of untreated urine sample with the identification of 3MH, 1MH, His and creatinine (Crea). Separation electrolyte: 500 mM HAc + 20 mM TRIS + 0.1% HEC (w/w) pH 3.4. Capillary: inner diameter 75 μm, effective length 56.5 cm, total length 65 cm; hydrodynamic injection 150 mbar s; separation voltage +30 kV, electric current 40 μA.

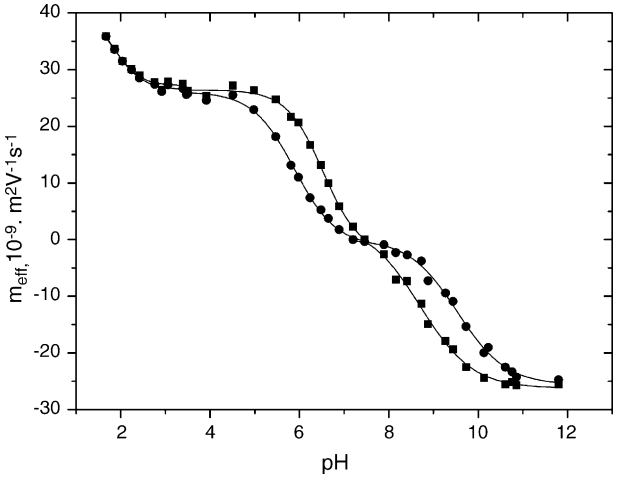

Fig. 5. Experimental dependence of the effective mobility of 3MH (●) a 1MH (■) on pH. Full lines were obtained by fitting the experimental data to Eqs. (2)–(4).

adding HEC. This is an electrically neutral polymer, which is being commonly used as a component of the separation buffer to suppress and stabilize EOF, and to prevent the undesirable adsorption of analytes on the capillary wall surface by forming a protective layer [19,26]. Urine contains a series of substances, in particular proteins, which adsorb on the capillary wall and deteriorate the analysis. A complicating factor was the presence of hydroxonium co-ions exceeding in mobility the methylhystidine cations. This difference in mobility led to a high electrodispersion of the electrophoretic zones and to the deterioration of the shapes of the corresponding peaks. Therefore, the composition of the separation electrolyte was modified further by adding TRIS, the effective mobility of which at the given pH is close to that of the analyte ions. On adding TRIS, the electrodispersion was significantly lowered and the peaks became symmetric. Optimum composition of the separation electrolyte comprised 0.5 M HAc, 20 mM TRIS and 0.1% (w/w) HEC, pH 3.4. The use of this electrolyte led to the efficient separation of His, 3MH, 1MH and creatinine down to the baseline, cf. Fig. 4.

Since only four major peaks appear in electropherogram of untreated urine sample, the urinary concentrations of 3MH, 1MH, His and creatinine are apparently higher than those of other organic cations, e.g. aminoacids and proteins in their cationic forms.

3.2. Evaluation of the dissociation constants

The effective mobility m_{eff} was evaluated from the measured migration times of the analyte and the marker of EOF, t_{mig} and t_{eof}, respectively, by using the equation

$$m_{\mathrm{eff}} = \frac{L_{\mathrm{t}} L_{\mathrm{d}}}{U}\left(\frac{1}{t_{\mathrm{mig}}} - \frac{1}{t_{\mathrm{eof}}}\right) \quad (1)$$

where L_{t} and L_{d} represent the total length of the capillary and its length to the detector, respectively, and U is the applied separation voltage. Migration times of 3MH and 1MH were measured in the system containing only one of these analytes and the EOF marker. The average value of the effective mobility was calculated from the results of three independent measurements at the selected pH. At pH < 2, the rate of the EOF has been already so low that the neutral EOF marker did not reach the detector even after 60 min of analysis. Therefore, an excess pressure of 5 mbar was applied at the capillary input to accelerate the transport of the EOF marker. In order to eliminate the effect of the fluctuation of the excess pressure, these measurements were repeated several times, and the average value of the effective mobility was calculated from three values of the migration time, which differed less than 2%. The dependences of the effective mobility on the solution pH are shown in Fig. 5.

The apparent equilibrium constants K'_{a1}, K'_{a2} and K'_{a3} characterizing the dissociation steps described in Fig. 2 were obtained by fitting the experimental dependence of the effective mobility m_{eff} on pH to the relationship, which is valid in the pH range close to $\mathrm{p}K'_{\mathrm{a}}$ of the corresponding dissociation step. At $\mathrm{pH} \approx \mathrm{p}K'_{\mathrm{a1}}$, $\mathrm{p}K'_{\mathrm{a2}}$ or $\mathrm{p}K'_{\mathrm{a3}}$, respectively, these relationships are given by the Eqs. (2)–(4), where m_{i+} is ionic mobility of corresponding ionic form of aminoacid [21–24]:

$$m_{\mathrm{eff}} = \frac{m_{\mathrm{AH_3^{2+}}} - m_{\mathrm{AH_2^{+}}}}{1 + 10(\mathrm{pH} - \mathrm{p}K'_{\mathrm{a1}})} + m_{\mathrm{AH_2^{+}}} \quad (2)$$

$$m_{\mathrm{eff}} = \frac{m_{\mathrm{AH_2^{+}}}}{1 + 10(\mathrm{pH} - \mathrm{p}K'_{\mathrm{a2}})} \quad (3)$$

$$m_{\mathrm{eff}} = \frac{m_{\mathrm{A^{-}}}}{1 + 10(\mathrm{p}K'_{\mathrm{a3}} - \mathrm{pH})} \quad (4)$$

Since the ionic strength was kept constant, the effective mobility is an explicit function of one variable, which is pH of

Table 1
Determinated values of apparent and thermodynamic dissociation constants for all dissociation levels of both methylated derivates of histidine

Compound	pK'_{a1}	pK'_{a2}	pK'_{a3}	pK_{a1}	pK_{a2}	pK_{a3}
3MH	1.86 (0.30)	5.85 (0.02)	9.54 (0.04)	1.66 (0.30)	5.79 (0.02)	9.60 (0.04)
1MH	1.81 (0.30)	6.45 (0.02)	8.82 (0.04)	1.61 (0.30)	6.39 (0.02)	8.88 (0.04)

Standard deviation in parentheses.

Table 2
Parameters of linear calibration curves for determination of 3MH, 1MH, His and creatinine (Crea) in water solution

Parameters	3MH	1MH	His	Crea
Sensitivity (mAU min L g^{-1})	523.0 (3.8)	489.8 (3.1)	596.4 (2.2)	573.6 (15.9)
Intercept (mAU min)	−0.3 (0.4)	−0.3 (0.3)	−0.2 (0.2)	4.4 (18.2)
Tested concentration range (mg mL^{-1})	1–200	1–200	1–200	100–2000
R	0.99995	0.99996	0.99999	0.99923
LOD (mg L^{-1})	0.4	0.5	0.4	–
LOD (μM)	2.4	3.0	2.6	–

Calibration curves were constructed from four different concentrations, each concentration was measured three times. LOD was estimated as concentration that gives the signal equal to three times the noise level, the standard deviations are shown in parentheses.

the separating electrolyte. Thermodynamic equilibrium constants K_a for the three dissociation steps can be then obtained by using the relationship (5):

$$pK_{ai} = pK'_{ai} - \log \frac{\gamma_{AH_{3-i}^{(2-i)+}}}{\gamma_{AH_{4-i}^{(3-i)+}}} \quad (5)$$

where $i = 1$, 2 or 3. The activity coefficients γ_i were estimated by using the McInnes's approximation to the Debye–Hückel Eq. (6), where z_i is the charge of the corresponding ion and I is the ionic strength of solution.

$$-\log\gamma_i = \frac{0.5085 z_i^2 \sqrt{I}}{1 + 1.5\sqrt{I}} \quad (6)$$

Apparent and thermodynamic equilibrium constants are summarized in Table 1.

3.3. Determination of 3MH and 1MH in a model aqueous mixture and in urine samples

The CE method was first tested by measuring the model aqueous samples of 3MH, 1MH and His in a range of their concentrations typically found in urine. The limit of detection (LOD) was estimated as the average concentration corresponding to S/N = 3 from four independent measurements at the wavelength of 214 nm. Parameters of the linear regression analysis are summarized in Table 2. The values of LOD found in the present study are comparable with those reported for low-molecular weight species in untreated urine with direct UV detection [27,28].

By using this method, 3MH, 1MH and creatinine were determined in 30 urine samples in three groups of human individuals. Peaks of 3MH, 1MH and His were identified first by successive spiking the urine sample with the standard solution in 10 μM steps, and second by comparing UV absorbance spectrum of their standard solutions with those of the urine samples, as measured both by the diode array detector of the CE instrument. The results of this analysis are summarized in Table 3. Although the average concentrations of 3MH in all three groups of individuals are comparable, a significant difference between the group of healthy individuals and the two groups of patients are revealed, when 3MH levels are correlated with 1MH data. Indeed, 3MH/1MH ratio is 0.41, 0.66 and 1.5 for healthy individuals, patients with diabetes mellitus II and obese patients, respectively, which indicates that a large part of 3MH metabolite in urine of healthy individuals comes from diet. Consequently, the 3MH levels in patients, either obese or with diabetes mellitus, appear to be relatively enhanced with respect to those in healthy individuals. This enhancement is likely to be a consequence of a more extensive degradation of muscle proteins in patients.

Table 3
Levels of 3MH and 1MH in urine of three different groups of individuals with the standard deviation given in parentheses

	3MH	1MH
Patients with diabetes mellitus		
Concentration (mg mL^{-1})	0.030 (0.017)	0.045 (0.066)
Concentration range (mg mL^{-1})	0.006–0.064	0.007–0.235
Concentration per 1 mg Crea (mg)	0.034 (0.008)	0.047 (0.052)
Concentration range per 1 mg Crea (mg)	0.022–0.047	0.009–0.168
Number of individuals	10	10
Obese individuals		
Concentration (mg mL^{-1})	0.041 (0.024)	0.028 (0.026)
Concentration range (mg mL^{-1})	0.014–0.083	0.007 – 0.090
Concentration per 1 mg Crea (mg)	0.031 (0.005)	0.021 (0.013)
Concentration range per 1 mg Crea (mg)	0.025–0.043	0.006–0.051
Number of individuals	10	10
Healthy individuals		
Concentration (mg mL^{-1})	0.032 (0.011)	0.078 (0.111)
Concentration range (mg mL^{-1})	0.022–0.058	0.005–0.403
Concentration per 1 mg Crea (mg)	0.038 (0.013)	0.089 (0.111)
Concentration range per 1 mg Crea (mg)	0.021–0.069	0.007–0.396
Number of individuals	10	10

4. Conclusions

3MH and 1MH can be efficiently separated in both acidic and alkaline media as cation and anion, respectively. Measurements of the effective mobility over a broad range of pH (1.67–11.80) has allowed us to evaluate the unknown dissociation constants of carboxylic, amino and side-chain imidazole groups in both 3MH and 1MH. A CE method for determination of the urinary 3MH and 1MH has been developed, which also makes it possible to determine simultaneously creatinine for elimination of diuresis. Optimum composition of the separation electrolyte comprised 0.5 M HAc, 20 mM TRIS and 0.1% (w/w) HEC, pH 3.4. The estimated limits of detection of 2.4 and 3.0 μM for 3MH and 1MH, respectively, correspond to the sensitivity currently achieved in electrophoretic separations with UV detection. An application of this method in analysis of untreated urine collected from the healthy individuals, and patients hospitalized with obesity or diabetes mellitus II has revealed the significant differences in 3MH/1MH ratio, which nevertheless have to be verified in a larger group of individuals. Essential advantage of the method is that it offers a fast determination of 3MH and 1MH in untreated urine, which in clinical analysis is an important aspect.

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