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Determination of urinary hippuric acid by micellar electrokinetic capillary chromatography

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Abstract

We propose a method for the simultaneous determination of hippuric acid (HA) and creatinine based on capillary micellar electrokinetic chromatography. Experimental conditions were 20 m*M* sodium phosphate, pH 7.20, 25 m*M* sodium dodecyl sulfate, 5% (v/v) acetonitrile. Electropherograms evidenced HA and creatinine peaks in less than 12 min. The method showed good linearity for both analytes and satisfactory within-day precision. The present method, which is accurate, sensitive, rapid and simple, may be applied to single-spot urine samples.

Keywords: Hippuric acid

1. Introduction

Hippuric acid (HA) is biosynthesised from glycine, benzoic acid and CoA by enzymes located in the mitochondrial matrix of liver and kidney cells [1]. This metabolic pathway provides an alternative flux of nitrogen from the usual urea precursors (ammonia, alanine and glutamate) to glycine [2] and it is utilised for the detoxification of aromatic compounds, such as toluene [3]. HA is therefore a physiological component of human urine, filtered in the glomerula and excreted by proximal tubular cells. HA excretion, that is modified in renal failure [4], contributes to total acid excretion, since it stimulates P-independent glutaminase localised at the proximal luminal membrane, with the formation of ammonia from glutamine [5].

Despite the numerous metabolic functions of HA, its quantification in urine has the disadvantage of showing great variation in and between individuals, depending on environmental and individual factors [6]. Thus, HA quantification in urine actually is mainly used as a diagnostic marker of exposure to toluene [7].

Urinary HA determination has been performed by high-performance liquid chromatography (HPLC) and gas chromatographic methods [8–10], by ^{1}H nuclear magnetic resonance (NMR) spectroscopy [11,12], by capillary electrophoresis [13–16], iso-

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tachophoresis [17] and also by capillary micellar electrokinetic chromatography (MEKC) [18]. The aim of this study was to develop and optimise a method based on capillary MEKC that allows one to detect and simultaneously quantify HA and creatinine. Quantification of the latter metabolite is important as internal standard used to normalise the concentration of HA for two reasons. It permits one to overcome errors of injection volume due to differences in sample viscosity and to exclude intra-day variability of HA renal excretion due to the variability of glomerular filtration rate.

2. Experimental

2.1. Reagents

All reagents were of analytical grade. Sodium phosphate and creatinine were from Farmitalia–Carlo Erba (Milan, Italy), Sodium dodecyl sulfate (SDS) from Fisher Scientific Company (Fair Lawn, NJ, USA), NaOH from J.T. Baker (Deventer, The Netherlands) and HA from Sigma (St. Louis, MO, USA). Electrophoretic buffers were prepared with ultra-pure grade water. 5-mm NMR tubes (Wilmad Glass, Buena, NJ, USA) were used to record ^{1}H-NMR spectra. Deuterium oxide containing 0.75% (w/w) of sodium-3-(trimethylsilyl)-[2,2,3,3,-$^{2}H_4$]-1-propionate (TSP) was used as solvent for NMR determinations.

2.2. Urine samples

Thirteen normal subjects (seven males and six females, age: 40±10 years) from the laboratory technical staff and eight subjects with creatinine clearance levels lower than 50 ml/min, from outpatients of our Hospital, were enrolled after informed written consent. All subjects collected spot urine samples in the morning.

Urine samples were analysed with dipsticks for routine chemical determination of pH (N-Multisticks AMES Bayer Diagnostics). Creatinine determination was made by the Jaffè method on a Hitachi 917 instrument. Urine samples were stored at −20 °C, until required for MEKC analysis and they were in any case processed within a month. After thawing, urine samples were centrifuged at 5000 rpm for 5 min, one volume of the supernatant was diluted with three volumes of ultra-pure grade water and the resulting solution submitted to MEKC analysis.

2.3. Experimental procedure

The capillary electrophoretic apparatus employed was a P/ACE system 2100 instrument (Beckman, Palo Alto, CA, USA) provided with the Gold Series 711 system (Beckman) for automated apparatus control and data acquisition and equipped with a UV absorbance detector. The bare silica capillary dimensions were 57 cm (50 cm to the detection window)× 75 μm I.D. (Beckman).

MEKC runs were performed at 25 °C in 20 m*M* phosphate buffer, that was prepared starting from NaH_2PO_4 and adjusting pH with NaOH. Then, SDS to a final 25 m*M* concentration was added. Experiments were performed applying a 25 kV voltage. In order to ensure current stability and high peak resolution, capillary was washed after each run with 1 *M* HCl for 4 min, followed by water for 5 min, then 0.5 *M* NaOH for 5 min and finally water for 5 min. Before starting, the separation capillary was rinsed with running buffer for 3 min. Sample injections were performed by the pressure fill method for 3 s with the anode electrode coincident to the capillary injection terminal. The small disturbance on the baseline observed when the sample plug crosses the detection window was used to determine the electroosmotic flow velocity. Analytes were detected at 214 nm. Peaks of creatinine and HA were confirmed by spiking with standards. The effects of the running buffer pH and acetonitrile (CH_3CN) percentage were studied evaluating the performance of the analysis at three different pH values (6.4, 7.2 and 7.7) and two different concentrations of CH_3CN (5, 10%, v/v). Standard curves were generated plotting the nominal concentration of standard solutions, expressed as m*M*, versus the measured area corrected (A_{corr}) according to the following formula [19]:

$$A_{corr} = A_m \cdot (l/t) - 1.26hd_r$$

where A_m is the measured area, t is the migration

time of the peak, l is the length of the capillary from the injection space to the spectrophotometric window, h is the peak height and d_r is the linear vertical dimension of the window (200 μm in our system). These data treatment allowed to express peak area in spatial instead of temporal dimension in order to correctly quantify analytes, when different mobilities are observed. A 4.0 m*M* standard solution of HA and a 10.0 m*M* standard solution of creatinine were used to prepare five scalar dilutions in the range 0.05–1.0 m*M* and 0.25–5.0 m*M*, respectively. The explored ranges were chosen to cover the physiological ranges of HA and creatinine considering the dilution factor 1:4 used in the sample preparation. Thus, linearity of the method for the two analytes corresponds to the urinary concentration range 0.2–4.0 m*M*, for HA and 1.0–20.0 m*M*, for creatinine. Each standard solution was analysed three times. The detection limit of HA and creatinine was defined as concentrations corresponding to peaks high three times the baseline noise.

2.4. ^{1}H-NMR procedure for correlation study

^{1}H-NMR spectra were registered at 25 °C by a Varian Gemini 300 apparatus (Varian, Palo Alto, CA, USA) operating at 300 MHz, following a standardised protocol [11]. Briefly, urine was added (9:1, w/w) to D_2O containing 7.5 g/kg TSP, that was used as chemical shift reference (0.0 ppm). Peaks were assigned in view of the chemical shift relative to TSP and the spin–spin coupling patterns. The assignments were confirmed by adding standard materials to the samples. Metabolite quantification was performed using the peak height at 7.48 ppm corrected for the two protons contributing to the resonance and normalised in relation to the creatinine signal. Thus, the obtained value expresses the mmol of HA per mol of creatinine present in the sample.

2.5. Statistics

Results are expressed as mean values±SD. Student's *t*-test was used to compare paired data and *P*-values <0.05 were considered statistically significant.

3. Results

We analysed a urine sample at three different pH values (6.4, 7.2 and 7.7) chosen considering urine pH physiological range in the presence of 5% (v/v) acetonitrile (Fig. 1). Peak of HA (A, migration time = 9.42 min) was resolved from other peaks at pH 7.2 and 7.7 and creatinine peak (B, migration time = 4.12 min) was well resolved at pH 7.2. To test the effect of the organic solvent concentration, the same urine sample was analysed at pH 7.2 in the presence of 5 and 10% (v/v) acetonitrile (Fig. 2). Best results were obtained with 5% of acetonitrile and these conditions were chosen to perform the following experiments. Electroosmotic flow, determined under our experimental conditions on 23 electrophoretic runs, was 3.98±0.08 min. Results of urinary creatinine obtained by capillary electrophoresis were compared to those obtained by the routine

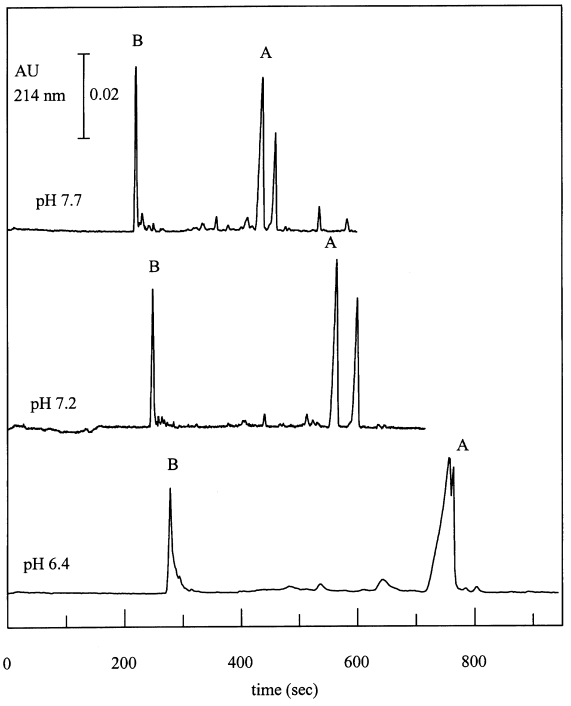

Fig. 1. MEKC of one-spot urine sample. Runs were performed at 25 °C in 20 m*M* sodium phosphate buffer at pH 6.4, 7.2 and 7.7, 25 m*M* SDS, 5% (v/v) CH_3CN. Capillary length was 57 cm, applied voltage 25 kV. Substances were revealed by UV absorbance detection. Peaks pertaining to hippuric acid (A) and creatinine (B) are evidenced.

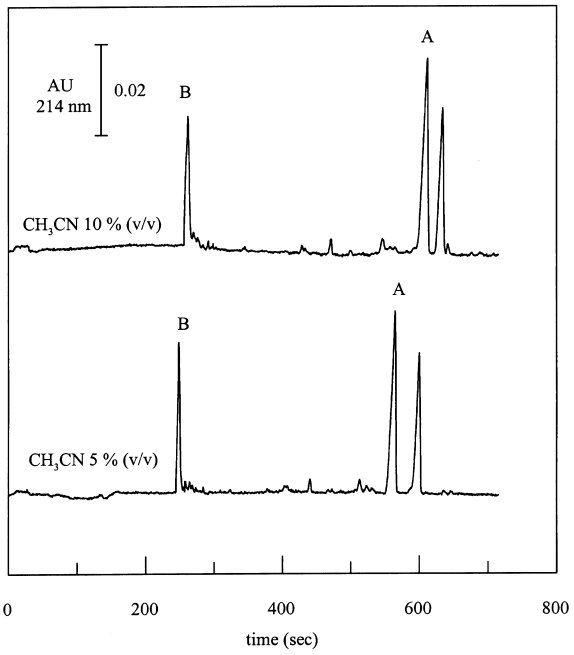

Fig. 2. MEKC of one-spot urine sample. Runs were performed at 25 °C in 20 m*M* sodium phosphate buffer, pH 7.2, 25 m*M* SDS, 5% and 10% (v/v) CH_3CN. Capillary length was 57 cm, applied voltage 25 kV. Substances were revealed by UV absorbance detection. Peaks pertaining to hippuric acid (A) and creatinine (B) are evidenced.

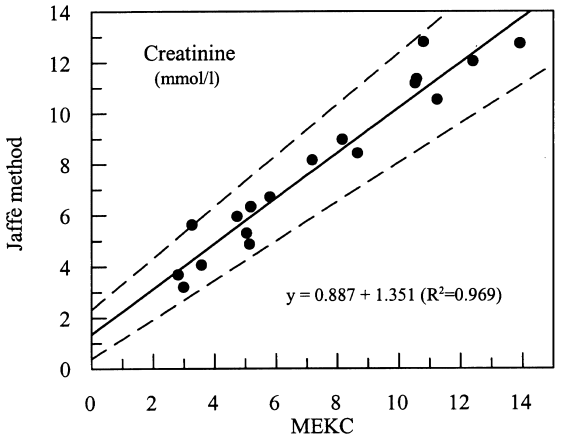

Fig. 3. Comparison between urinary creatinine concentrations determined by MEKC and by Jaffè method. 95% Confidence limits (dashed lines) were: 0.768–1.005 (slope) and 0.388–2.314 (*y*-intercept).

method. A good correlation between the two analytical approaches was found (Fig. 3, $P=0.015$), thus peak area of creatinine were used to normalise HA concentrations, that were expressed as mmol/mol creatinine. In order to validate the MEKC method we checked the linearity of the assay using standard solutions of HA and creatinine at five concentrations, which covered the physiological ranges. A good linear relationship in the range 0.05–1.0 m*M* for HA (peak area $=25.21x+0.12$ m*M*, $r=0.993$) and 0.25–5.0 m*M* for creatinine (peak area $=20.09x+1.88$ m*M*, $r=0.998$) was observed. In order to take into account possible interference due to the biological matrix, calibration curves were repeated adding standards to a urine sample diluted 1:4. No significant differences were observed between curves obtained with standard and those obtained with urine plus standard. The detection limit of HA defined as a peak still measurable (peak to noise ratio ≥ 3) was 12.0 μmol/l. Reproducibility as reflected by within-run precision data, evaluated determining six times six different samples, is reported in Table 1. The higher RSD (12.2%) was observed in a sample with very low HA concentration (39.2 mmol/mol creatinine). The same samples were stored at 4 °C and analysed on 3 consecutive days. They showed RSD values lower than 15%.

In order to compare HA data obtained by the proposed method with data obtained by another analytical approach, we analysed 21 urine samples (13 from normal subjects and eight from patients with pathological creatinine clearance values) also by ^{1}H-NMR spectroscopy. The two methods showed a good linear regression, but the minimum quantitation limit of MEKC was lower than that of NMR

Table 1
Within-day precision of the method

Sample	HA/creatinine (mmol/mol)	RSD (%)
1	363.0	6.9
2	394.5	8.0
3	204.3	5.8
4	39.2	12.2
5	285.0	5.9
6	398.3	5.8

Each result, expressed as HA/creatinine, corresponds to the mean value of six determinations.

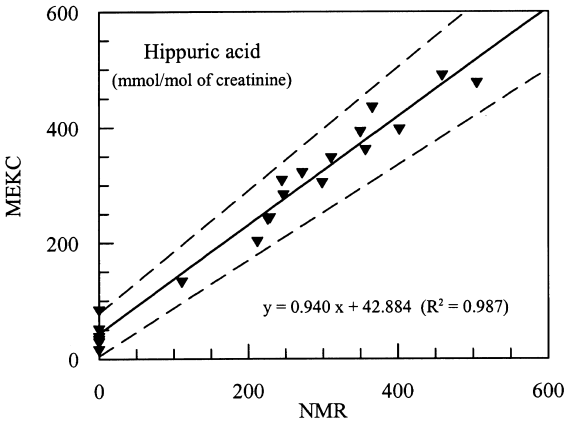

Fig. 4. Comparison between HA concentrations (mmol/mol of creatinine) determined by ^{1}H-NMR spectroscopy and by MEKC on 21 urine samples, 13 from normal subjects and eight from patients with pathological creatinine clearance values. 95% Confidence limits (dashed lines) were: 0.827–1.064 (slope) and 4.17–77.82 (y-intercept).

spectroscopy method under our analytical conditions (Fig. 4).

4. Discussion

In the present study we validated a method for the simultaneous determination of urinary HA and creatinine based on capillary MEKC. The theoretical principles of analytical electrophoresis and of capillary MEKC have been described by Kleparnik and Bocek [20]. In this study, the analytical protocol was defined with regard to pH value and to the percentage of the organic solvent acetonitrile in order to obtain the better reproducibility and resolution of HA and creatinine peaks in urine samples. The organic solvents are used in order to increase solubility, to reduce the electroosmotic flow velocity, to diminish retention factors, by reducing distribution constants, while variation of pH principally affects resolution due to the impact of pH on dissociation of weak electrolytes. The proposed method was tested for accuracy and precision, in general use. The recovery study was omitted since, analysing the sample without pre-treatment, a loss of the analyte was not expected. The simplification of procedural steps greatly improves the turnaround time and represents one of the major advantages of our method with respect to HPLC and GC methods. NMR spectroscopy allows analyse urine samples without pretreatment, but this technology showed a lower sensitivity. Finally, the MEKC method is also convenient for general use since it permits the contemporaneous determination of HA and creatinine. In this way it is possible to analyse single spot urine samples, without considering diuresis. It should be outlined that, although the use of HA as an indicator of occupational exposure to toluene is contradictory, the biological exposure index (BEI) to toluene is defined as the ratio HA/creatinine [21].

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