Short communication

Determination of urinary orotic acid and uracil by capillary zone electrophoresis

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Abstract

We describe a simple method for measuring orotic acid and uracil concentration in urine by capillary zone electrophoresis in 20 mM Na-borate buffer, pH 9.2. The method was applied for studying a patient with HHH (hyperornithinemia, hyperammonemia and homocitrullinuria) syndrome. A high value of uracil excretion was found during periods of relatively low orotic acid excretion and normal ammonemia. The orotic acid level in urine was increased by increasing protein intake. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Ortic acid; Uracil

1. Introduction

The size of the pyrimidine pool in humans is regulated through a balance among de novo anabolic, salvage and catabolic pathways [1,2]. The de novo synthesis initiates from cytosolic carbamoylphosphate and involves orotic acid, which is phosphoribosylated to orotidine-5′-monophosphate (OMP) and converted to all the other required pyrimidine nucleotides. The degradation proceeds largely through the formation of uracil and thymine that are oxidised in the presence of dihydropyrimidine dehydrogenase and then hydrolysed to carbamyl-β-amino acids.

Abnormally high urinary levels of orotic acid and uracil have been found in some defects of urea cycle, which cause accumulation of carbamoylphosphate and, thus, stimulate de novo pyrimidine synthesis, i.e., ornithine transcarbamylase deficiency, citrullinemia, argininemia [3], and HHH (hyperornithinemia, hyperammonemia and homocitrullinuria) syndrome [4]. Orotic aciduria in formiminotransferase/cyclodeaminase deficiency [5] and in lysinuric protein intolerance [6] has been also attributed to increased pyrimidine synthesis. Inherited deficiency of orotate phophoribosyltransferase and OMP decarboxylase causes orotic aciduria without increasing uracil excretion [7], while dihydropyrimidine dehydrogenase deficiency leads to increased excretion of uracil and other pyrimidine bases [8].

The colorimetric methods for determining orotic acid [9] and uracil [10] in the urine are affected by the presence of interfering compounds that must be removed by chromatographic techniques [11]. Gas chromatography and high-performance liquid chromatography methods are more sensitive [12–15], but

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they require rather complicated sample preparations in order to obtain accurate results [16]. Capillary electrophoresis was employed for the determination of orotic acid [17–20], while this method failed to show an increase in uracil excretion in patients with ornithine transcarbamylase deficiency, citrullinemia or HHH syndrome. An enzymatic method based on the coupled reactions catalysed by orotate phosphoribosyltransferase and OMP decarboxylase was described for the determination of orotic acid in biological fluids [21].

We report here a simple and rapid method for quantitative determination of both orotic acid and uracil in the urine by capillary zone electrophoresis (CZE). The method is applied successfully to study a patient with HHH syndrome.

2. Experimental

Orotic acid and uracil were purchased from Sigma (St. Louis, MO, USA). Water and other reagents were high-purity commercial samples from Sigma and Merck (Darmstadt, Germany). All solutions were used without filtration. Urine samples were collected from healthy volunteers and from patient with HHH syndrome and stored at $-20°C$. Urinary homocitrulline concentration in untreated patient (a 21-month-old boy) was 51.7 mmol/mol creatinine; plasma concentrations of ammonia and ornithine were 90 μM (reference range 10–35 μM) and 652 μM (reference range 20–136 μM), respectively.

Unless otherwise stated, urine samples were diluted in 10 volumes of water. Because of the low solubility of uracil in the biological samples, we carefully dissolved, before the analysis, any precipitate present in the urine [17]. Sample dilution as well as alkalinization by NaOH (final concentration 0.1 mM; pH~12) facilitated uracil solubilisation [22].

The samples were injected into a Beckman P/ACE electrophoresis system by using a positive pressure at 0.5 p.s.i. for 5 s (1 p.s.i.=6894.76 Pa). Separations were performed in a fused-silica capillary [57 cm (50 cm effective length to the photometer)×75 μm] at relatively high electroosmotic flow, i.e., 20 mM Na-borate buffer, pH 9.2 at 30°C, so that all compounds were carried toward the cathode and UV monitored. The applied potential was increased in 12 s (as

suggested by the Beckman's operation manual) to the final running voltage of 30 kV, where the potential was held constant for the duration of the analysis (4 min). The data were collected at 5 Hz frequency and stored on the hard disk of a personal computer. The capillary was flushed sequentially for 4 min with 100 mM NaOH, water and separation buffer between each run. A volume flow of about 15 nl/s was observed by dissolving acetone in the separation buffer as marker [23].

Glutamine was derivatised with phenylisothiocyanate and assayed spectrophotometrically after purification by reversed-phase chromatography [24]. Creatinine was assayed by the method of Jaffé [25]. Ammonia concentration was determined enzymatically [26].

3. Results and discussion

Electropherograms of diluted urine samples from patient with HHH syndrome and healthy control subject are shown in Fig. 1. All the 280-nm absorption profiles showed a large peak around 2.5 min that was mainly due to the presence of uric acid in the samples. In the case of patient, two major extra signals were noticed at 2.0 min and 3.2 min. The peaks had the same migration times and the same absorption spectra, in the 250–350 nm range, of authentic uracil and orotic acid (Fig. 2). Interfering unidentified chromophores were observed by analysing the spectra at shorter wavelengths. The migration-time reproducibility ranged from 2 to 5%, while the variation coefficient of the peak areas was less than 5% (n=6) by repeating the measurements both on a single day and on different days with different electrolyte batches. The repeatability of analysis did not increase significantly by using the uric acid peak on the electropherograms as internal standard. Concentrated urine samples (osmolality>400 mOsm/kg) showed faster migration times and worse peak-separation quality. Control experiments obtained by dissolving the analytes into diluted normal urine samples showed linear relationships between the peak areas and the concentrations of uracil (r=0.9847; n=10) and orotic acid (r=0.9935; n=10) in the range from 10 μM to 1 mM. The limits of detection of uracil and orotic acid (with a signal-to-
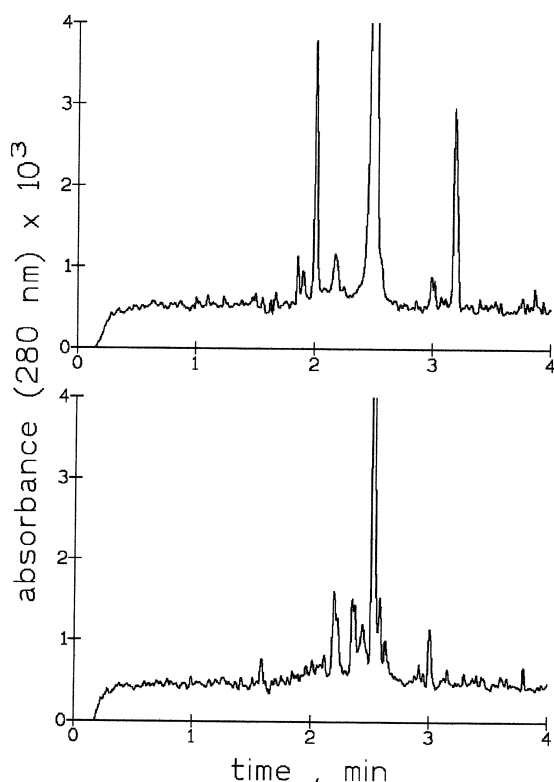
Fig. 1. Electropherograms of urine samples from patient with HHH syndrome (top) and healthy control subject (bottom). Patient's urine contained 0.106 mol orotic acid per mol creatinine and 0.140 mol uracil per mol creatinine. The samples were diluted in 10 volumes of water before being injected. Profiles display absorption at 280 nm. All other conditions were as described in the text.
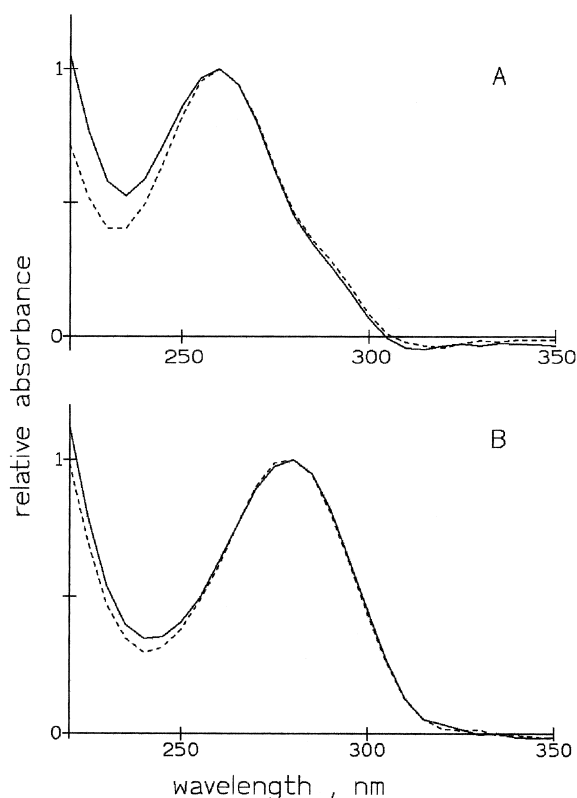

Fig. 2. Diode-array analysis of the major extra signals present in the electropherograms from the patient with HHH syndrome (see Fig. 1). Broken lines represent the spectra of authentic standards. The 2.0-min peak is compared with uracil standard (A), while the 3.2-min peak is compared with orotic acid standard (B).

noise ratio of 3) were 5 μM and 2.5 μM, respectively.

As shown in Table 1, the patient with HHH syndrome excreted a high value of uracil during period of relatively low orotic acid excretion and normal ammonia level. Urinary orotic acid excretion increased markedly by increasing protein intake and paralleled the changes in plasma glutamine, which is considered the best guide to effective therapy in patients with inherited hyperammonia [27]. This result is in line with the findings on subjects with ornithine transcarbamylase deficiency [15], suggesting that evaluation of increased flux in the pyrimidine biosynthetic pathway by urinary orotic acid and uracil analysis may be a reliable method for studying the inborn errors of urea synthesis.

The sensitivity of our method for the determination of orotic acid is similar to that reported by Ševčík et al. [20], who utilised a low pH buffer in the anionic mode to eliminate interference of non-orotate compounds with analysis. In this paper, we show that orotic acid measurements can be achieved also at higher pH in the cationic mode, provided that the urine samples are diluted, all the precipitates [17] are carefully solubilized, and the detector is fixed around 280 nm. Under these conditions, because of the higher background electrolyte pH, we were able to detect both orotic acid and uracil in the same urine sample. The reliability of the procedure was demonstrated by following the metabolic changes in a patient with HHH syndrome. In conclusion, we believe that our method for the determination of orotic acid and uracil in urine is rapid and simple and

Table 1
Plasma ammonia and glutamine levels and urinary orotic acid and uracil excretion in the patient with HHH syndrome, who has been challenged with small changes in diet to assess protein tolerance

Protein intake (g/kg/day)	Ammonemia (μM)	Glutaminemia (μM)	Orotic aciduria (mol/mol creatinine)	Uraciluria (mol/mol creatinine)
1.36	28	614	0.023	0.103
1.50	31	813	0.106	0.140
Reference values	10–35	330–810	<0.003	<0.004

can be useful for screening patients with metabolic defects as well as for studying the relationship between pyrimidine biosynthetic pathway and urea cycle in humans.

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