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Diagnosing AICA-ribosiduria by capillary electrophoresis

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Abstract

AICA-ribosiduria is a recently discovered inherited metabolic disease caused by a defect in final steps of purine *de novo* biosynthesis—5-amino-4-imidazolecarboxamide ribotide (AICAR)-transformylase/inosinemonophosphate (IMP)-cyclohydrolase (ATIC). A rapid and selective capillary electrophoretic method for screening of patients with AICA-ribosiduria is described. The method is based on direct ultraviolet detection of 5-amino-4-imidazolecarboxamide (AICA) and 5-amino-4-imidazolecarboxamide riboside (AICAr) in untreated urine. Background electrolyte consists of 100 mM malonic acid adjusted with γ-aminobutyric acid (pH 2.7). Under the given separation conditions both compounds of interest are well separated from other substances with separation efficiency of 1 020 000 and 130 000 theoretical plates/m for AICA and AICAr, respectively. Total analysis time is 3 min with the limits of detection of 3.6 μM and 4.5 μM for AICA and AICAr, respectively. The usefulness of the presented method for screening of patients with ATIC deficiency is demonstrated on samples of Chinese hamster ovary cell line defective in ATIC activity, spiked urine samples and urine samples from patients treated with high-dose MTX which do not excrete increased amounts of AICA and AICAr compared to untreated controls ($p < 0.05$). The described method is fast and effective enough for diagnostic applications.

Keywords: Inborn errors; Purines; Metabolism; Screening

1. Introduction

Inherited diseases of purine metabolism are almost exclusively related to salvage pathways. Eighteen different defects of purine metabolism have already been documented in the salvage pathways [1]. Defects of purine metabolism are characterised by abnormal concentrations of substrates of defective enzymes and/or their metabolites in cells or body fluids.

In purine *de novo* synthesis (PDNS) two defects have been long time known—adenylosuccinate lyase (ADSL) deficiency (McKusick 103050) [2,3] and phosphoribosylpyrophosphate synthetase (PRPPS) superactivity (McKusick 311850) [4,5]. The 5-amino-4-imidazolecarboxamide (AICA)-ribosiduria (McKusick 608688) is third recently discovered [6] inherited metabolic disease caused by a defect in final steps of PDNS—5-amino-4-imidazolecarboxamide ribotide (AICAR)-transformylase/inosinemonophosphate (IMP)-cyclohydrolase (ATIC) (EC 2.1.2.3/3.5.4.10). The enzyme catalyses the formylation of 5-amino-4-imidazolecarboxamide ribotide (AICAR) to produce 5-formamino-4-imidazolecarboxamide ribotide (FAICAR) and the cyclization of FAICAR to IMP (Fig. 1). The first diagnosed patient suffered from severe neurological defect, congenital blindness and presented dysmorphic features. The disease is detectable by positive Bratton–Marshall test [7] and the detection of 5-amino-4-imidazolecarboxamide riboside (AICAr) – the dephosphorylated intermediate – in urine by HPLC [6].

Methotrexate (MTX), chemically *N*-[4-[[(2, 4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-glutamic acid, is an antifolate which is responsible for inhibition of a number of folate-dependent metabolic steps including AICAR-transformylase [8]. MTX is applied for the treatment of cancer, rheumatoid arthritis and other disorders [9–11], and reportedly [9,12] increases excretion of AICAr.

Capillary electrophoresis is a powerful technique in diagnosing purine and pyrimidine inherited metabolic defects [13–19]. We describe here the development and optimisation of the

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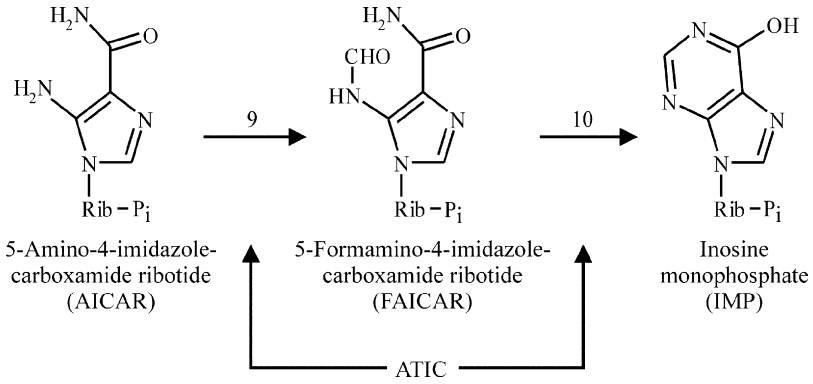

Fig. 1. Conversion of 5-amino-4-imidazolecarboxamide ribotide (AICAR) to inosinemonophosphate (IMP) via 5-formamino-4-imidazolecarboxamide ribotide (FAICAR) is catalyzed by bifunctional enzyme AICAR-transformylase/IMP-cyclohydrolase (ATIC).

method capable to analyse AICA-ribosiduria metabolites in human body fluids.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical reagent grade. AICA, ammonium hydroxide, ammonium acetate, citric acid, phosphoric acid, γ-aminobutyric acid (GABA), bases and nucleosides were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide and malonic acid were obtained from Lachema (Brno, Czech Republic). Calf intestine alkaline phosphatase (1 I.U./μl) was obtained from Fermentas (Hanover, MD, USA). AICAr was obtained from Toronto Research Chemicals (North York, ON, Canada). All solutions were prepared using deionised water (18.3 MΩ cm). The derivatized polyacrylamide boronate Affi-Gel® was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Methotrexate was obtained from Ebewe Pharma (Unterach, Austria).

2.2. Patients and samples

Urine samples from healthy volunteers were from 10 adults (Caucasians, Czech; 2 males and 8 females; age range, 24–55 years; mean, 35.8 years). MTX urines were from cancer patients (lymphosarcomatosis, $n=5$; refractory anemia, $n=2$; Non-Hodkin's lymphoma, $n=3$; Caucasians, Czech; 6 males and 4 females; age range, 28–59 years; mean, 42.4 years) treated with high-dose MTX (4.8–11 g/m^2 of body surface during 12 h). Urine samples were taken immediately after receiving therapeutic dose.

Chinese hamster ovary (CHO) cell line defective in ATIC activity (Ade-F) was kindly provided by Prof. David Patterson (University of Denver, USA).

2.3. CE apparatus

All assays were performed on a P/ACE 5510 with diode array detector (Beckman Instruments, Fullerton, CA, USA). The electrophoretic separations were carried out in an uncoated fused-silica capillary (50 μm I.D. × 375 μm O.D.; Polymicro Technologies, Phoenix, AZ, USA). The capillary had an effective length of 20 cm (total length 27 cm) and was operated at 25 °C. UV detection over the range of 190–300 nm (cartridge detection window 100 μm × 800 μm) was used. Samples were loaded by low-pressure injection (3.45 kPa) for 6 s. Capillary was washed at the beginning of each working day with deionised water, 0.1 M sodium hydroxide, water and separation buffer for 5 min; between runs it was washed with water for 1 min and separation buffer for 2 min. Separation buffers were filtered through a 0.45 μm membrane filter (Teknokroma, Barcelona, Spain) and sonicated for at least 5 min before use.

2.4. Purification of samples and stability of AICAr

Solid phase extraction cleaning procedure based on borate—*cis*-diol interaction was performed using the boronate-derivatised polyacrylamide gel [20]. Dry gel (0.5 g) was weighed into a plastic column and swelled in starting buffer (0.25 M ammonium acetate, pH 8.8). Prepared column had a capacity of 180 mg of inosine per gram of dry gel. Just 1 ml of sample (urine or cell medium) was applied onto the column in a purification process to not exceed its capacity. All used solutions were passed through the column by vacuum of 50 kPa.

2.4.1. Urine purification

Five millilitres of urine were purified using a column. Urine sample (1 ml) alkalinised with the starting buffer (4 ml) was applied onto column which was subsequently washed with the starting buffer (10 ml) and water (10 ml) for removing impurities. This procedure was repeated for each of 5 ml of urine sample. Retained compounds were eluted with 0.1 M acetic acid (5 ml), freeze-dried, reconstituted in deionised water (50 μl), analysed by CE or stored at −20 °C.

The recovery was measured using urine samples supplemented with AICAr. The mean recoveries ($n=6$ for each concentration) were 75.4 ± 1.8% (mean ± SD) and 67.2 ± 0.6% for 50 and 200 μM addition of AICAr.

2.4.2. Purification of cell medium

Half a ml of cell medium alkalinised with the starting buffer (2 ml) was applied onto column which was subsequently washed

with the starting buffer (10 ml) and water (10 ml) for removing impurities. Retained compounds were eluted with 0.1 M acetic acid (5 ml), freeze-dried, reconstituted in deionised water (50 μl), analysed by CE or stored at −20 °C.

We investigated the stability of AICAr in water and urine at −20 °C, 4 °C and room temperature. Fifty microlitres of standard solution of AICAr (1 mM) were added to 450 μl of urine and water, and analysed periodically.

2.5. Dephosphorylation of ribotides

Purified calf intestine alkaline phosphatase was applied for the conversion of nucleotides to nucleosides: 40 μl of liquid sample were mixed with 5 μl of reaction buffer (0.1 M Tris–HCl, 0.1 M $MgCl_2$, pH 7.5). Deionised water was added up to total volume of 49 μl. This mixture together with 1 μl of alkaline phosphatase solution (1 I.U.) was incubated for 2 h at 37 °C and immediately analysed or stored at −50 °C.

3. Results and discussion

3.1. Optimisation of CE system

Low pH (<3.0) of background electrolyte (BGE) was chosen because of p*K* of imidazole part of target compounds is 3.23 [21] and a number of urinary impurities is also reduced at low pH [17]. The first parameter optimised was composition of BGE (Fig. 2) with an anion concentration of 100 mM. Separation in sodium citrate (p*K*s 3.1, 4.7, 5.4) system (pH 2.5) appeared to be most suitable with respect to analysis time (<2.0 min). However, separation efficiency of tested analytes (441 000 and 77 000 theoretical plates/m for AICA and AICAr) was poor (Fig. 2a). Sodium phosphate (p*K*s 2.1, 7.2, 12.3) buffer (pH 2.5) allowed application of maximally 15 kV because of high-generated current (migration time 2.12 min and >4.0 min for AICA and AICAr, respectively) with insufficient efficiency (Fig. 2b, 197 000 and 41 000 TP/m for AICA and AICAr, respectively). Sodium malonate (p*K*s 2.8, 5.67) system (pH 2.5) was suitable with respect to analysis speed (Fig. 2c) and separation efficiency was 1 087 000 and 100 000 TP/m for AICA and AICAr, respectively. Assays in malonic acid adjusted with GABA (pH 2.5) gave much better efficiency for AICA (1 500 000 TP/m) than previous system, provided "double-buffering" (p*K*s of GABA 4.0) and generated lower current. Therefore, 100 mM malonate-GABA buffer, pH of 2.5, +25 kV (Fig. 2d) was selected for further optimisations.

The second optimised parameter was pH of BGE and its influence on the separation efficiency and analysis time (Fig. 3). Analyses performed at pH ≤ 2.5 were fast, but peak splitting of AICAr was observed (Fig. 3a and b). The pH of 2.7 was chosen as a compromise (Fig. 3c). Final conditions consisting of 100 mM malonic acid titrated with GABA at pH of 2.7, +25 kV with a voltage ramp of 1 min and the rate of the detector set at 4 Hz allowed analysis of AICA and AICAr within 3.0 min with separation efficiency of 1 020 000 and 130 000 TP/m, respectively.

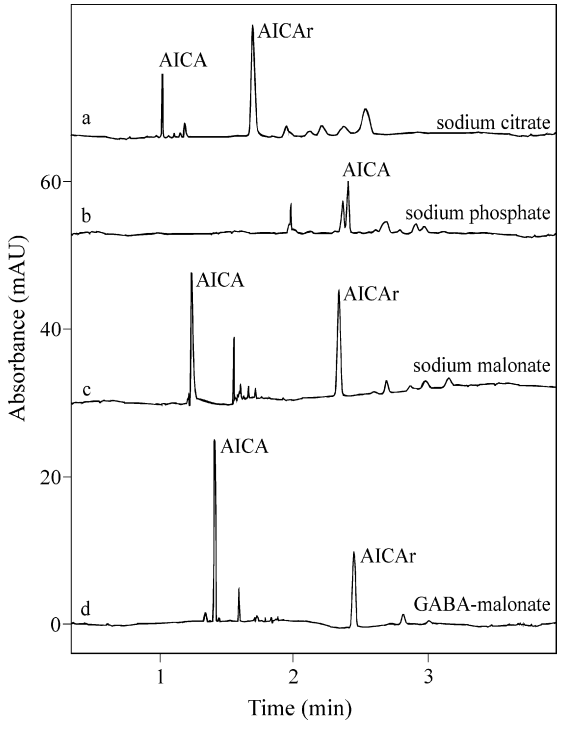

Fig. 2. Influence of buffer composition on CE analyses of spiked urines with mixture of AICA and AICAr. Separation conditions: (a) sodium citrate, pH 2.5, +30 kV, 88 mA; (b) sodium phosphate, pH 2.5, +15 kV, 90 mA; (c) sodium malonate, pH 2.5, +25 kV, 105 mA; (d) GABA-malonate, pH 2.5, +25 kV, 88 mA; anion concentration of 100 mM, detection wavelength 265 nm.

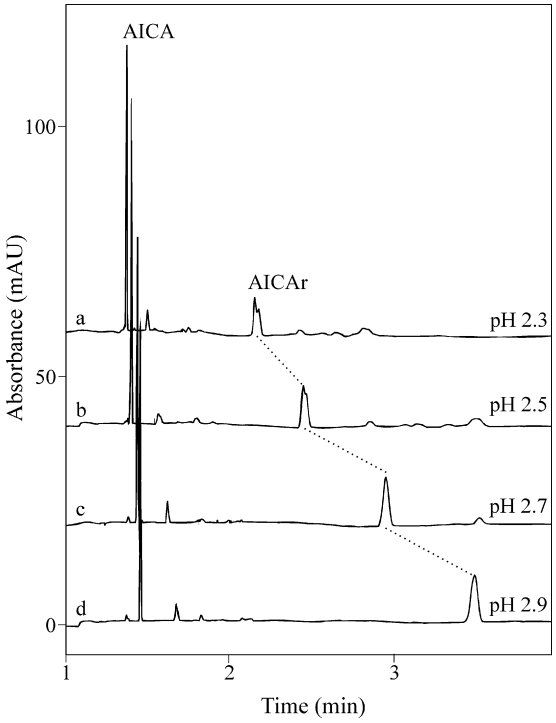

Fig. 3. CE analyses of spiked urines with mixture of AICA and AICAr at pH 2.3–2.9. At lower pH (2.3 and 2.5) splitted peak of AICAr was observed. Conditions: 100 mM GABA-malonate, +25 kV, detection wavelength 265 nm.

3.2. Validation data

Both compounds of interest were detectable at concentration of 3.6 μM and 4.5 μM (limit of detection with a signal/noise ratio of 3) for AICA and AICAr, respectively. The method was linear in the concentration range of 5–500 μM ($r > 0.999$) for both the compound. The intra- and inter-day imprecision of corrected peak area (CV, $n = 10$) for AICA was 6.8 and 9.0% for 50 μM addition, 4.4 and 7.7% for 300 μM addition. CVs for AICAr were 3.4 and 7.8%, 1.5% and 6.7%, respectively. The reproducibility of the migration times was measured on the mixture of target compounds in six consecutive runs. Run-to-run ($n = 20$) and day-to-day ($n = 6$) CVs were 0.4 and 1.2% for AICA and 0.9 and 4.5% for AICAr. The recovery of the compounds was measured using urine samples supplemented with target compounds (added concentrations, 50 and 150 μM). The mean recoveries ($n = 6$ for each concentration) were 94.2 ± 7.7% (mean ± SD) and 99.9 ± 2.2% for AICA and 96.8 ± 2.5% and 99.1 ± 1.8% for AICAr.

AICAr was stable in urine and water at 4 °C (decrease <3% within 30 days) and −20 °C (no decrease observed in 30 days). Half-life at room temperature was 10 and 6 days for water and urine, respectively. AICAr was decomposed to AICA.

3.3. Analysis of ATIC defective CHO cell line and human urine

Potential usefulness of the method was demonstrated on CHO cell line defective in ATIC activity (assigned Ade-F line, see Fig. 1) and spiked urine samples. Ade-F cells similarly to situation in human ATIC deficiency [6] accumulate large amounts of first substrate of defective enzyme—AICAR. We used alkaline phosphatase for its conversion (see Section 2.5) into riboside (AICAr). CE analyses of intracellular content, culture medium and standard mixture of target compounds with UV spectra are shown in Fig. 4. CE analysis of intracellular content confirmed the presence of AICAr (1.15 pM/cell) in the cells only after alkaline phosphatase treatment indicating that a vast majority of the accumulated substrate is intracellularly in a nucleotide form. AICAr was also detectable in cultivation medium (Fig. 4d) after 10-fold preconcentration using boronate gel.

CE analyses of healthy urine samples spiked with AICA and AICAr showed no significant interferences with target compounds (Fig. 3). Therefore, we applied the developed CE method on urine samples from patients treated with MTX ,where an accumulation of AICAr and/or its metabolite (AICA) was expected (Fig. 5) contrary to earlier report [12,22]. Neither AICAr nor AICA were detected.

Hence, cleaning procedure using boronate gel was applied on both healthy and MTX urine for their 100-fold preconcentration. CE analyses of preconcentrated urines are shown in Fig. 6. AICAr was found not only in MTX urine (Fig. 6a) but also in healthy urine (Fig. 6b) after preconcentration procedure. This fact was confirmed by CE analysis of both MTX urine (not shown) and healthy urine (Fig. 6c) spiked with AICAr before preconcentration procedure. CE analyses of urines in malonate-GABA using the boronate gel for their preconcen-

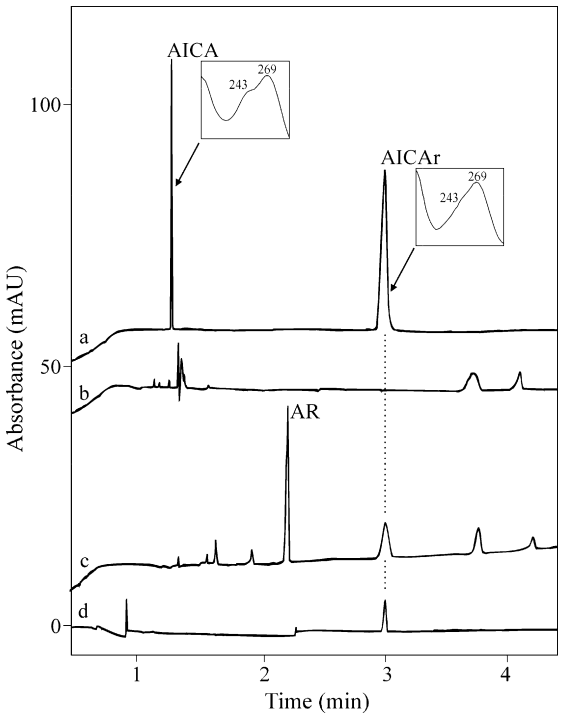

Fig. 4. CE analyses of standard mixture (a), Ade-F line of CHO cells before (b) and after alkaline phosphatase (c), purified Ade-F media of CHO cells (d) with UV spectra of target compounds (insets). See Section 2.4 for more details. Separation conditions: 100 mM GABA-malonate, pH of 2.7, +25 kV, detection wavelength 265 nm. AR, adenosine.

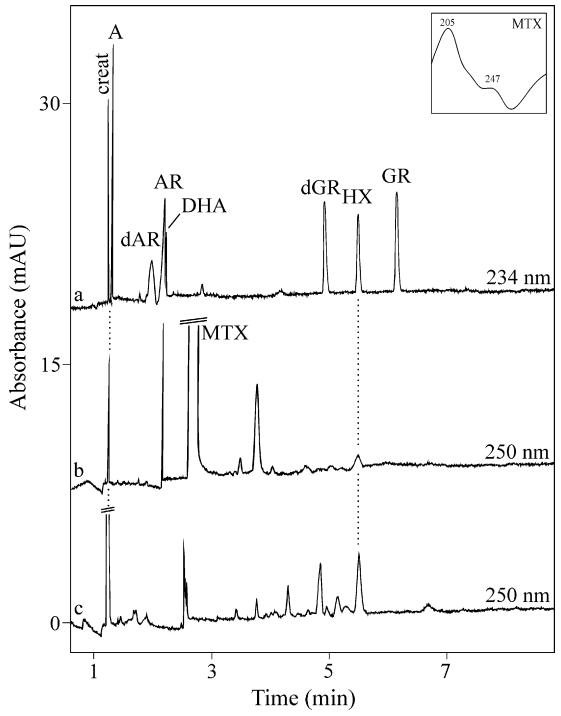

Fig. 5. CE analyses of standard mixture of purine bases and ribosides (a), MTX urine (b) and healthy urine (c). UV spectrum of MTX (inset). Conditions: 100 mM GABA-malonate, pH 2.7, +25 kV, inj. 6 s. Creat, creatinine; A, adenine; AR, adenosine; dAR, deoxyadenosine; DHA, dihydroxyadenine; GR, guanosine; dGR, deoxyguanosine; HX, hypoxanthine; MTX, methotrexate.

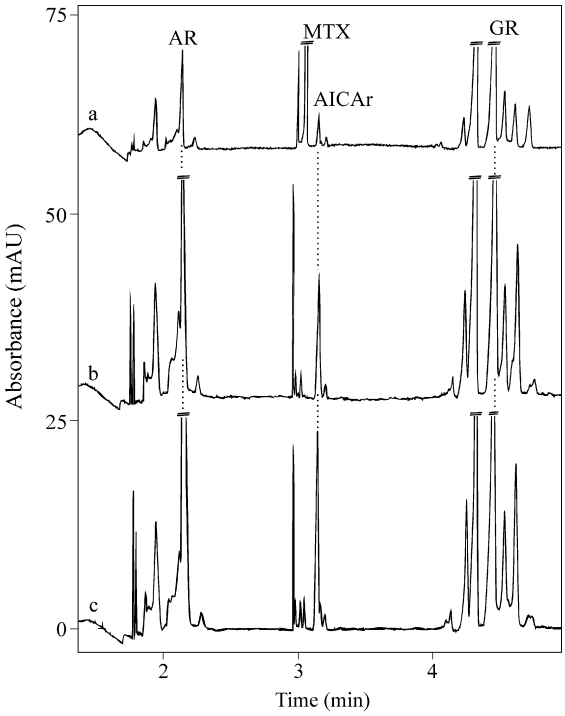

Fig. 6. CE analyses of purified MTX urine (a), healthy urine (b) and healthy urine with added AICAr (c). Conditions: 100 mM GABA-malonate, pH 2.7, +25 kV, inj. 6 s, detection wavelength 265 nm. AR, adenosine; GR, guanosine; MTX, methotrexate.

tration confirmed the common presence of AICAr in human urine. Although an increased excretion of AICAr in urine of MTX treated patients compared to controls was reported previously [9,22], we found no significant difference ($p < 0.05$, AICAr excretion 0.26 ± 0.11 μM (mean ± SD) and 0.25 ± 0.15 μM in healthy and MTX urine, respectively).

4. Conclusion

We have developed an effective CE method for diagnosing a recently discovered inherited metabolic disorder—AICA-ribosiduria [6]. AICA and AICAr can be identified from native urine directly without any pretreatment. Short analysis time, sufficient sensitivity and simplicity are basic benefits of this method. AICAr is commonly present in healthy human urine and can be determined by the method after preconcentration using boronate gel. An accumulation of AICAr was not confirmed in urine samples of patients treated with high-dose MTX compared to controls.

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