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Screening method for inherited disorders of purine and pyrimidine metabolism by capillary electrophoresis with reversed electroosmotic flow

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Abstract

Capillary electrophoresis with electroosmotic flow reversed by cationic surfactant for diagnosis of purine and pyrimidine inherited enzyme deficiencies is reported. Final separation conditions consist of 45 m*M* borate, 55 m*M* *N*-tris[hydroxymethyl]methylglycine, 10 m*M* tartrate, 1 m*M* cetyltrimethylammonium bromide and 0.44% tetrabutylammonium hydroxide-2-amino-2-methyl-1,3-propanediol (pH 8.6). Average sensitivity (2.51 μ*M*), reproducibility of migration times (run-to-run C.V.≤0.6%, day-to-day C.V.≤2.5%), linearity (R^2>0.994) and imprecision (mean intra-assay RSD 4.7% and inter-assay RSD 6.6%) of the method are acceptable for diagnostic purposes. Applicability of the method is demonstrated on urine samples from patients with enzymatically proven enzyme deficiences.

Keywords: Purine; Pyrimidine

1. Introduction

Inherited defects of purine and pyrimidine (P&P) metabolic pathways are associated with serious, sometimes fatal, clinical consequences [1]. Purine and pyrimidine species play heterogeneous roles in the control of many cellular functions, and so the spectrum of clinical symptoms is very broad. The majority of these defects are associated with neurological abnormalities, but urological problems, disturbances in drug metabolism, life-threatening immunodeficiencies, anemias and cancers are also common clinical symptoms. Moreover, as in other types of metabolic disorders, the clinical manifestation of each particular disease can be quite variable making clinical selection of patients a difficult task. This explains the need for fast, automated and inexpensive diagnostic methods that enable examination of a large number of samples. Methods must be powerful and capable of identifying known as well as novel disorders.

Purine and pyrimidine species were first measured a century ago (see Ref. [2] for review). The first use of anion exchange in 1949 [3] introduced chromatographic techniques into this field. Liquid chromatography has dominated in routine diagnostic processes until now [1,4–7]. Cheaper, but more laborious and

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time-consuming, 2D TLC [8] has also been used. Recently, two novel technologies (HPLC–electrospray ionization-mass spectrometry [9] and ^{1}H-NMR [10]) have been successfully utilized for diagnostic purposes.

Beginning with early isotachophoretic approaches [11,12], electromigration techniques have been used for separation of P&P species in the past [13–20]. The use of capillary electrophoresis (CE) as a diagnostic tool for particular P&P inherited diseases [21–24] as well as generally [25] has been reported during the last few years.

We report here alternative conditions, utilizing separation in alkaline background electrolyte (BGE) with electroosmotic flow (EOF) reversed to the same direction as the separated species. The conditions reported speed up the diagnostic process and also serve as an alternative tool for cases of complicated biological matrices.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent grade. Boric acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Bases, nucleosides, 2-amino-2-methyl-1-propanol (Amp), 2-amino-2-methyl-1,3-propanediol (Ampd), 3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid (CAPSO), *N*-tris[hydroxymethyl]methylglycine (TRICINE), tetrabutylammonium hydroxide (40% solution, TBAH), cetyltrimethylammonium bromide (CTAB) and other chemicals were obtained from Sigma (St. Louis, MO, USA). Deionised water (18 MΩcm) was used for preparation of all solutions.

2.2. Subjects and samples

Urine samples from healthy controls were from 200 children (Caucasians; 118 males and 82 females of the Czech Republic; age range, 1–15 years; mean, 4.2 years). The urines from patients (all from UK population) were from persons with enzymatically proven P&P enzyme deficiencies. All samples were obtained as 40-μl urine spots on filter paper and were dissolved in 150 μl of deionised water, dried under a stream of nitrogen and redissolved in 40 μl of deionised water.

Seven spot urine specimens from healthy children (creatinine range, 1.1–15.1 m*M*; mean, 4.7 m*M*) were used for measuring imprecision and reproducibility of migration times. All samples were centrifuged (3000 *g*, 5 min) before loading.

2.3. CE apparatus and separation conditions

All experiments were performed on P/ACE 5510 with diode array detector (Beckman Instruments, Fullerton, CA). The electrophoretic separations were carried out in an uncoated fused-silica capillary (50 μm I.D.×375 μm O.D., Polymicro Technologies, Phoenix, AR, USA). The capillary had an effective length of 40 cm (total length 47 cm) and was operated at 30 °C. Analyses were run at 30 kV. UV detection was over the range 190–300 nm using a cartridge detection window of 100×800 μm. The data rate of the detector was set at 4 Hz. Samples were loaded for 8 s by low-pressure injection (0.5 p.s.i.). At the beginning of each working day the capillary was washed with water, 0.1 *M* sodium hydroxide, water and separation buffer for 5 min. Between runs the capillary was washed with water (0.3 min), 0.1 *M* sodium hydroxide (0.3 min) and running buffer (1 min) (see Imprecision section for details). These standard conditions were used for all experiments. Faster separations were carried out with the use of capillary, which had an effective length of 20 cm (total length of 27 cm). Analyses were run at 25 kV and detected using detector data rate of 16 Hz (see below).

A total of 14 bases and nucleosides (the key diagnostic metabolites) together with two major urinary UV-absorbing constituents (creatinine and hippuric acid) were taken as the target group of compounds. Stock solutions of tested compounds and a standard mixture were prepared by dissolving compounds in boiling deionised water under sonification.

Initially, BGE consisting of 60 m*M* borate–Na (pH 9.0) with varying CTAB concentration (0, 0.01, 0.02, 0.05, 0.1, 0.3, 0.6, 1.0, 2, 5 m*M*) was used for determination of required CTAB concentration which gives reproducible reversed EOF. Acceptable

results were obtained at the concentration of 1 m*M* of CTAB and above.

Furthermore, 60 m*M* borate (pK_a of boric acid 9.2) titrated with Ampd (pK_b 8.8) and Amp (pK_b 9.7) over the pH range 8.3–10.0 was used to determine optimal pH for the separation of the tested mixture. All conditions failed to separate adenine and thymine and besides, deoxyadenosine migrates with the EOF. It is not easy to separate adenine and thymine under alkaline conditions because of similar p*K*s [20]. Additions of acetonitrile and methanol (up to 20%) were tested in order to separate adenine and thymine. Acetonitrile at a concentration higher than 15% resolved the species but overall separation substantially worsened. On the basis of previously observed interactions with adenine derivatives [26] tartaric acid was tested. Addition of 10 m*M* tartaric acid fully resolves adenine and thymine with no other substantial effect on the separation. An ion-pairing reagent TBAH (0.44%) was successfully used to force deoxyadenosine from the EOF region. Other coions (*N*,*N*-bis(2-hydroxyethyl)glycine, pK_a 8.3; TRICINE, pK_a 8.1) in variable molar ratios to borate were used in order to spread the separation mixture across the separation window. Final conditions consisting of 45 m*M* borate, 55 m*M* TRICINE, 10 m*M* tartrate, 1 m*M* CTAB, 0.44% TBAH titrated with Ampd (pH 8.6) allowed baseline separation of all compounds of interest (Fig. 1). It is possible to separate the mixture using a 27-cm long (total length) capillary using a 16-Hz detector data rate (Fig. 1). Such fast data collection is necessary because fast migrating species (e.g. orotic acid) may be undetected and certain species may become apparently unresolved at lower values. Under these conditions, however, the detector produces a relatively noisy trace (all these electropherograms are presented in ''smoothed'' form (sum of traces±10 nm from optimal wavelength)) and we found it potentially risky during diagnostic evaluation. Selection of the maximal analytical conditions (capillary length and voltage) depends solely on the performance of the particular detector used. For all the following assays a 47-cm long capillary was used. The average efficiency of the separation was 213 000 theoretical plates. The slightly lower efficiency for inosine, guanosine and hypoxanthine can probably be attributed to electrodispersive phenomena in this

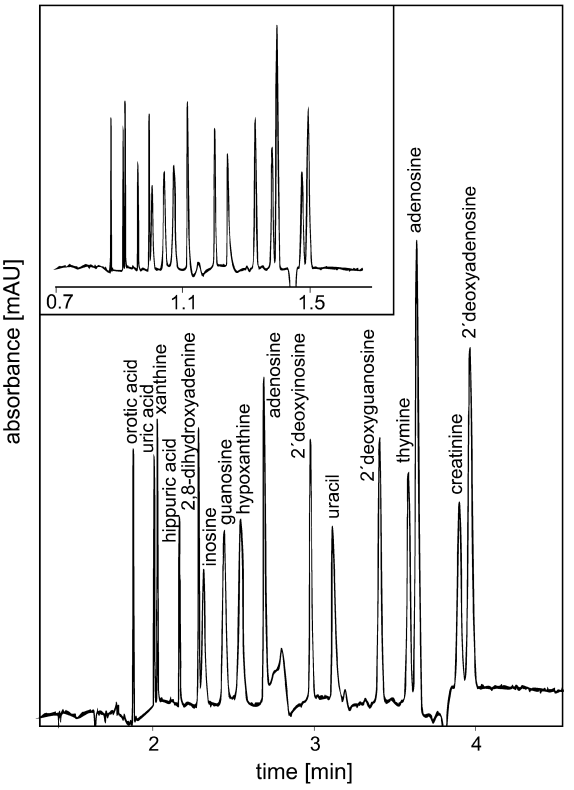

Fig. 1. Separation of mixture of purines and pyrimidines and major urinary constituents. Conditions: BGE: 45 m*M* borate, 55 m*M* TRICINE, 10 m*M* tartrate, 1 m*M* CTAB, 0.44% TBAH and Ampd (pH 8.6). Voltage 30 and 25 kV (upper left corner), capillary 40/47 and 20/27 cm (upper left corner), detection at 200 nm (''smoothed'': sum of traces 200±10 nm in upper left corner). For other conditions see Experimental section.

multiple-coion BGE [27,28]. The final BGE was stable at 4 °C for at least 1 month.

2.4. Diagnostic metabolites and interferences

Using the final separation conditions, migration and spectral properties of compounds of interest in diagnosing inherited metabolic disorders, common artifacts from medication [1], and several other UV-absorbing compounds were measured (Table 1). The neat compounds dissolved in deionised water were analyzed with the exception of succinyl-aminoimidazole carboxamide riboside (SAICAR) and succinyladenosine (SAR) where diagnostic metabolites are not commercially available.

Table 1
Characteristics of metabolites and possible interferences

Analyte	Effective mobility (10^{-9} m^2 V^{-1} s^{-1})	Absorbance maxima[a] (nm)
Phtalic acid	−26.45	<195
Orotic acid	−24.60	276, 203
Succinyladenosine	−22.08	270
Uric acid	−20.54	233, 290
SAICAR[b]	−19.49	267
Xanthine	−19.70	239, 274
Oxypurinol	−17.60	240, 265
Vanillylmandelic acid	−16.70	278
Homovanillic acid	−16.37	278
Argininosuccinic acid	−15.15	<193
Hippuric acid	−14.86	<225
1-Methylxanthine	−14.48	242, 273
2,8-Dihydroxyadenine	−13.41	300
3-Methylxanthine	−12.92	272
7-Methylxanthine	−12.80	274
Inosine	−11.87	248
Uridine	−10.89	261
Guanosine	−9.87	251
Pseudouridine	−9.13	262
Adenosine	−8.49	256
1-Methylhistidine	−8.09	<211
Hypoxanthine	−7.21	251
Histidine	−4.68	<205
2′-Deoxyinosine	−4.62	248
Phenylalanine	−4.61	<190
Tyrosine	−4.22	<221
3-Methylhistidine	−4.14	<195
Theophyline	−3.50	272
Uracil	−3.49	256
7-Methylguanine	−3.28	245, 282
Theobromine	−3.20	271
2′-Deoxyguanosine	−3.13	251
Adenine	−1.38	257
Carnosine	−1.15	<198
Thymine	−1.06	259
Urea	0.00	<190
Creatinine	0.00	231
2′-Deoxyadenosine	0.62	260

[a] Absorbance maxima in the range 220–300 nm. For compounds not exhibiting absorbance maxima in this range, upper value of absorbance is given (e.g. <220).
[b] SAICAR, succinylaminoimidazole carboxamide riboside.

2.5. Limit of detection, linearity and recovery

Limit of detection for compounds of interest was determined using 8-s injection (1.1% of the total capillary volume injected with the sample; Table 2). The linearity was tested by analyzing eight stock solutions in the concentration range 20–600 μ*M*. The method is linear (R^2>0.994) for all compounds of interest.

The recovery of the compounds was measured using urine sample spot extracts with two additions of standards. The mean recoveries for diagnostic

Table 2
Measurement of linearity and detection limit of studied compounds

Analyte	Linearity [$y = ax + b$]			Detection limit (μM)
	(a)	(b)	(R^2)	
Ade	19.325	58.49	0.9958	2.53
AR	20.305	−185.85	0.9947	6.32
dAR	17.580	108.56	0.9964	3.33
dGR	22.855	462.76	0.9977	0.62
DHA	16.696	102.25	0.9991	1.58
dHR	18.996	123.67	0.9992	1.53
GR	27.613	40.20	0.9996	3.04
Hipp	8.431	31.20	0.9996	3.69
HR	16.784	−110.03	0.9952	5.45
HX	29.782	102.99	0.9976	1.05
OA	10.453	61.77	0.9997	2.26
T	27.807	266.25	0.9989	1.28
U	14.525	165.26	0.9985	1.75
UA	13.515	140.10	0.9987	1.71
X	16.342	157.78	0.9991	1.54

metabolites were 101.5% (RSD 11.2%) for 50 μM and 113.6% (RSD 8.0%) for 200 μM of added standards, respectively.

2.6. Imprecision

Imprecision of the method was tested by assaying seven samples of healthy volunteers containing three metabolites (uric acid, xanthine and inosine) over 10 consecutive days (Table 3).

Reproducibility of migration times was measured on the same set of samples of healthy volunteers (run-to-run C.V., 0.5, 0.6 and 0.6% ($n=10$) for uric acid, hypoxanthine and creatinine, respectively; day-to-day C.V., 1.4, 2.0 and 2.5% ($n=10$) for uric acid, hypoxanthine and creatinine, respectively). It should be noted that between-run washing of the capillary with sodium hydroxide is necessary for obtaining reproducible mobilities because omitting this step results in gradual decrease of electroosmotic flow mobility (1.3% between runs).

Table 3
Imprecision of method

Analyte	Mean (μM)	Intra-assay (RSD, %)	Inter-assay (RSD, %)
Hypoxanthine	71.7	5.5	9.7
Xanthine	35.4	5.5	6.1
Uric acid	1642.2	3.2	3.9

3. Results and discussion

We analyzed 200 urine samples from healthy children by this method. A typical electropherogram of a urine sample from a healthy infant is shown in Fig. 2. Urine from healthy infants contained dominant peaks of uric acid, creatinine, hippuric acid and usually varying amounts of hypoxanthine, xanthine, pseudouridine, and uracil. The usefulness of the method for diagnostic purposes was demonstrated on urine samples from patients suffering from inherited disorders of P&P metabolism. The diagnostic metab-

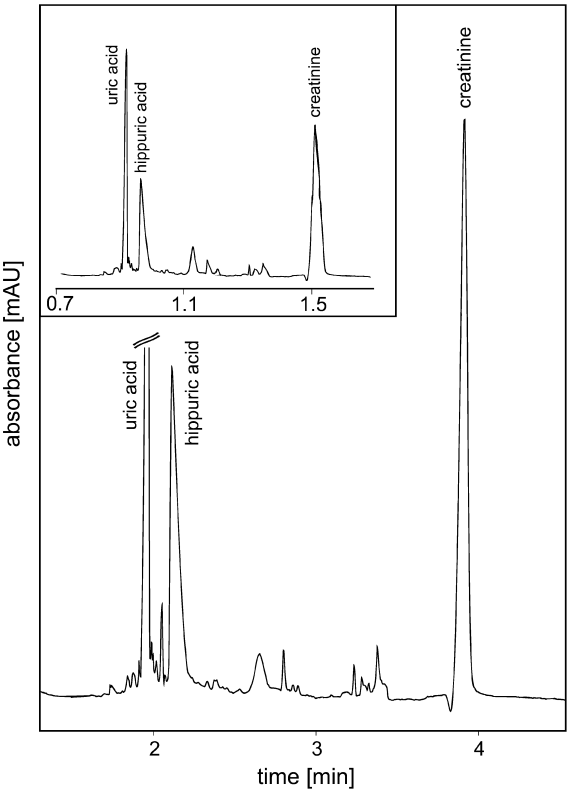

Fig. 2. Electropherogram of urine from healthy infant. Conditions as in Fig. 1.

olites were identified by migration times and spectral fit.

The analysis of urine from a patient with adenine phosphoribosyl transferase (APRT, EC 2.4.2.7) deficiency (Fig. 3) allowed identification of a 2,8-dihydroxyadenine peak (formed from accumulated adenine by xanthinoxidase). The electropherogram of urine from a patient with dihydropyrimidine dehydrogenase (DiPYDH, EC 1.3.1.2) deficiency revealed diagnostic peaks of uracil and thymine (Fig. 4). Dominant peaks of inosine, guanosine and their deoxyribosides were well resolved in the sample from a purine nucleoside phosphorylase (PNP, EC 2.4.2.1) deficient patient (Fig. 5). The sample from a patient with xanthine oxidase (XO, EC 1.2.3.2) deficiency revealed a dominant peak of xanthine migrating after a small peak of uric acid (Fig. 6). In the analysis of urine from patients with orotic acidurias due to ornithine transcarbamylase (OTC, EC 2.1.3.3; Fig. 7), orotate phosphoribosyltransferase (OPRT, EC 2.4.2.10) deficiency (data not shown) and patients with urea cycle deficiencies (data not shown), a fast migrating peak of orotic acid was observed. Patients with orotic acidurias can be easily verified by an alternative CE diagnostic method [24]. In the sample from an adenylosuccinate lyase (AMPSL, EC 4.3.2.2) deficient patient two fast migrating peaks were observed (data not shown). They were attributed to SAR and SAICAR (two dephosphorylated substrates of the enzyme) on the basis of UV-spectra and expected migration behavior. The method is advantageous for diagnosing diseases associated with overexcretion of metabolites with high effective mobility (AMPSL deficiency and orotic acidurias; in particular for diagnosing carriers of OTC deficiency with only slightly elevated OA in urine) which do not give very effective peaks when EOF is of opposite direction. In the urine from a

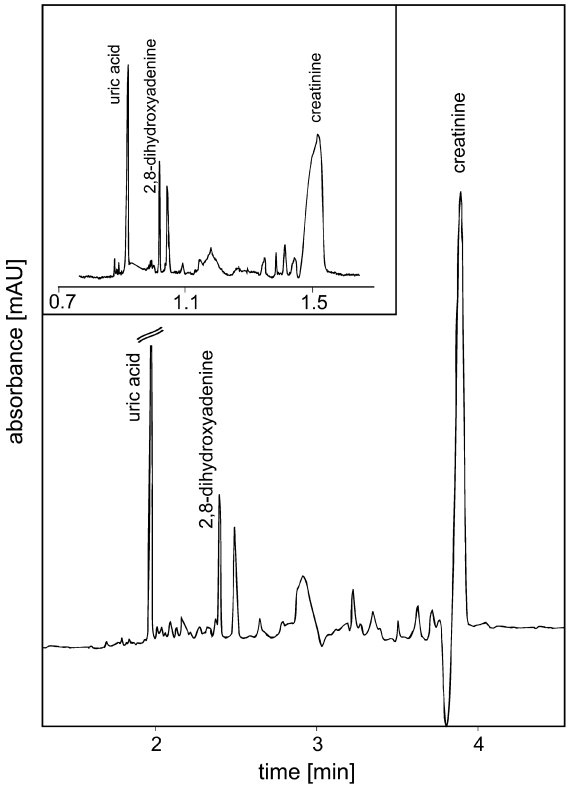

Fig. 3. Electropherogram of urine from patient with adenine phosphoribosyl transferase deficiency. Conditions as in Fig. 1.

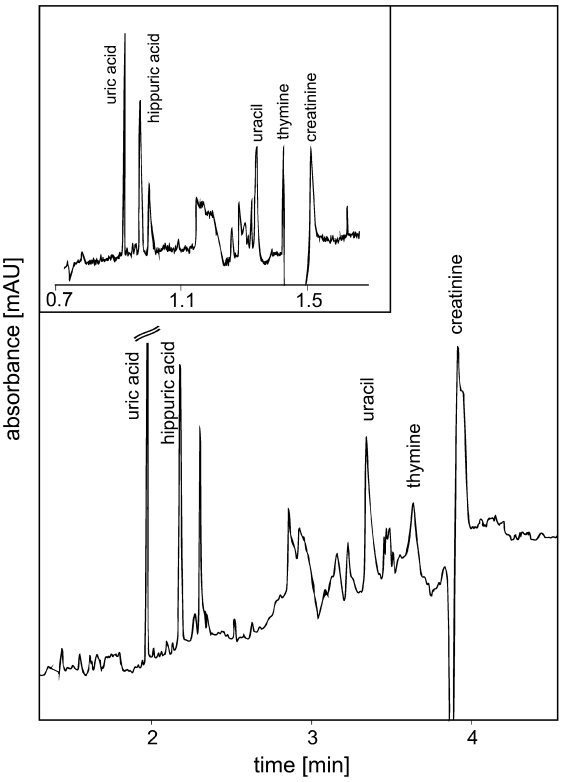

Fig. 4. Electropherogram of urine from patient with dihydropyrimidine dehydrogenase deficiency. Conditions as in Fig. 1.

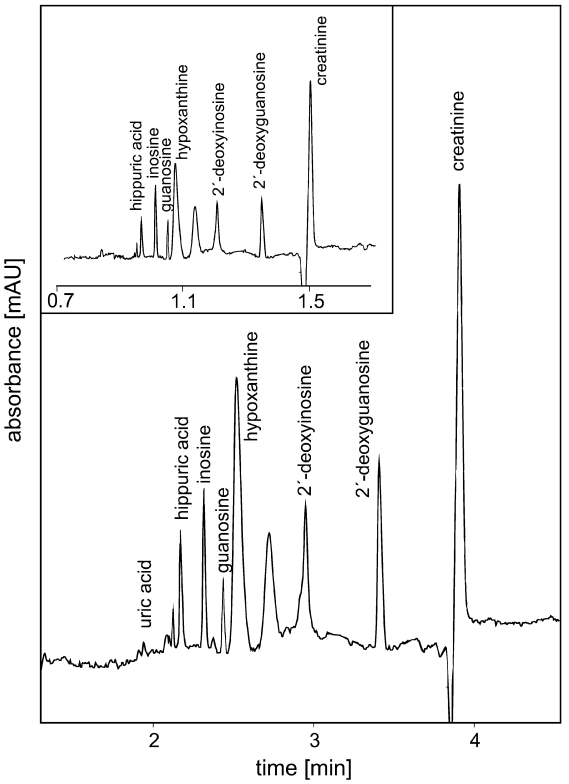

Fig. 5. Electropherogram of urine from patient with purine nucleoside phosphorylase deficiency. Conditions as in Fig. 1.

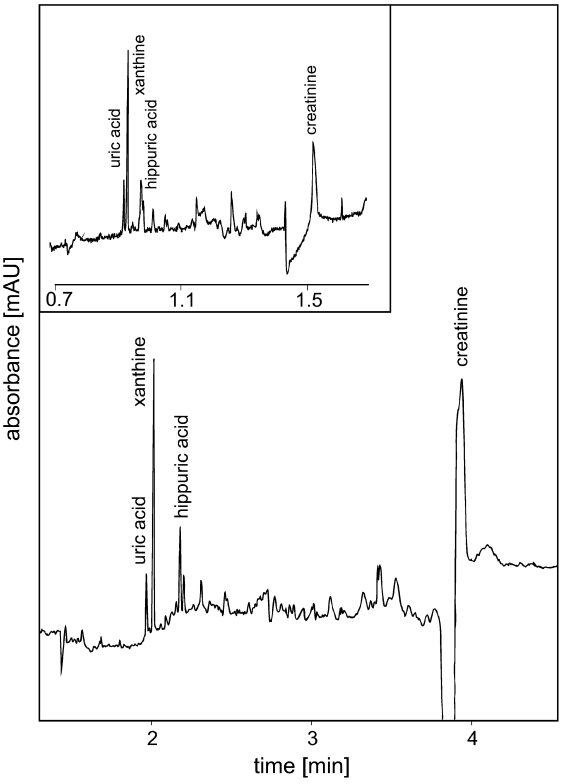

Fig. 6. Electropherogram of urine from patient with xanthine oxidase deficiency. Conditions as in Fig. 1.

patient with hypoxanthine phosphoribosyl transferase (HPRT, EC 2.4.2.8) deficiency only elevated peak of uric acid was observed (data not shown). Peaks of accumulated adenosine and deoxyadenosine were seen in the sample from a patient with adenosine deaminase (ADA, EC 3.5.4.4) deficiency (data not shown).

4. Conclusions

We report a method suitable for high-throughput screening of all purine and pyrimidine metabolic disorders associated with overexcretion of accumulated metabolites. The analysis time depends solely on performance of the detector and can be as short as 1.5 min. The method is routinely used in the author's laboratory as the first tier in diagnostic routine. Where analysis is inconclusive (typically due to abundant presence of drug metabolites) in some of the samples (~10–20% in our hands), the second approach [25] is used for clarification. This is also the case of pathological findings prior to enzyme assay confirmation of the diagnosis. As the methods use different pH and composition of BGE the selectivity towards diagnostic metabolites and potential interferences differ markedly.

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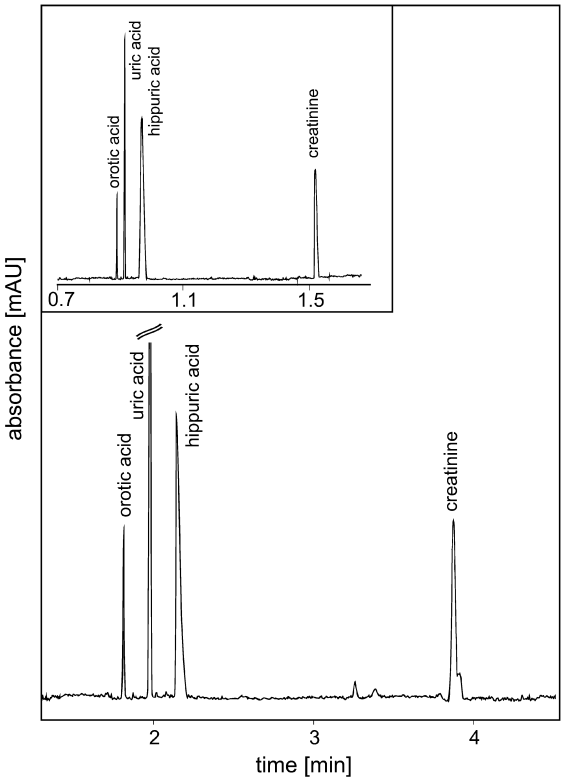

Fig. 7. Electropherogram of urine from patient with ornithine transcarbamylase deficiency. Conditions as in Fig. 1.

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