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Capillary electrophoresis for rapid profiling of organic acidurias

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Organic acids analysis is a powerful technique in the diagnosis of inborn errors of metabolism. Clinically, patients present with severe symptoms, and early detection and appropriate treatment are often lifesaving. Most of the existing methods are based on gas chromatography in combination with mass spectrometry and require sophisticated equipment and complex sample pretreatment and derivatization. We propose a rapid, simple, and automated capillary electrophoretic method for routine analysis of urine to detect 27 organic acids related to metabolic diseases. With this method, direct measurements are performed on samples after initial centrifugation and dilution, if needed. Separation is performed in pH 6.0 phosphate buffer with methanol added as an organic modifier, −10 kV applied potential, and ultraviolet detection at 200 nm. The assay is completed in <15 min, and alternative separation conditions are proposed in case of overlapping peaks. The developed method allows the identification and quantitation of methylmalonic, pyroglutamic, and glutaric acids in samples of patients with diseases related to these acids.

The analysis of organic acids in urine is a well-established procedure for the diagnosis of inherited disorders of amino acid and organic acid metabolism. The large number of organic acids in urine and the complexity of the mixture makes its separation and quantitation very difficult; gas chromatography–mass spectrometry is the most reliable technique for this purpose *(1)*. However, despite the number of methodologies described, they all require laborious sample pretreatment, expensive and sophisticated equipment, and highly qualified personnel; in fact, most laboratories spend a long time extracting, purifying, and derivatizing organic acids from urine before a patient sample is ready to be analyzed by gas chromatography–mass spectrometry.

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A rapid diagnosis of critically ill newborns who present with coma and metabolic acidosis is crucial to instituting the adequate therapy and avoiding fatal consequences.

Capillary electrophoresis (CE) is suitable for detecting important changes in the metabolic profiles of body fluids and provides a rapid and simple alternative to other techniques in routine analysis *(2–5)*.

A previous study separated and quantified nine short-chain organic acids *(6)*. The aim of the present study was to separate and identify more organic acids, including new groups of aromatic acids, amino acids, and keto acids with relevance for diagnostic purposes. Three different internal standards (ISs) have been proposed and evaluated, and the intra- and interassay precision for migration time have been studied. Therefore, a rapid and simple screening method for acidurias has been developed, and some pathologic samples have been tested. Methylmalonic, pyroglutamic, and glutaric aciduria were detected in pathological samples in which these metabolites showed increased concentrations, and the peaks were easily identified.

Materials and Methods

APPARATUS

Capillary zonal electrophoresis was performed on a Beckman System 5500 (P/ACE) equipped with an ultraviolet detector set at 200 nm, an automatic injector, and a 37-cm total length (75-μm i.d.), polyacrylamide gel-pretreated column cartridge.

All experiments were carried out at 25 °C. Sample injections were made by pressure for 5 s with an applied reversed voltage of 10 kV for buffer S and 15 kV for buffer A.

CHEMICALS

Calibrators. Calibrators and ISs were obtained from Sigma Chemical Co. Aminoadipic acid, ketoglutaric acid disodium salt, 3-hydroxybutyric acid sodium salt, *p*-hydroxyphenylpyruvic, oxalacetic, and glycolic acids were 98% minimum purity; lactic acid lithium salt was 97% mini-

mum purity. The rest of chemicals were analytical-reagent grade (>99% purity).

Buffers. Phosphoric acid (85%) was from Merck. Acetic acid and sodium hydroxide were from Panreac, and methanol was from Scharlau.

Buffer solutions and all dilutions were prepared with water purified by a MilliQ-System (Millipore). The electrophoretic buffer S, pH 6.0, contained 0.2 mol/L phosphate and 100 mL/L methanol *(6)*. The second buffer used in the study, buffer A, was 0.2 mol/L phosphoric acid and 0.01 mol/L acetic acid adjusted to pH 4.0 with sodium hydroxide and did not contain methanol.

SAMPLES

Fresh urine samples were collected from healthy and ill babies under 4 months and refrigerated at −20 °C. Pathological samples corresponded to methylmalonic, glutaric, and pyroglutamic acidurias. Before analysis, samples were diluted with water (1 volume of sample plus 2 volumes of water) and centrifuged for 3 min at 2000*g*.

Results and Discussion

As shown in electropherogram A in Fig. 1, 22 calibrators of different organic acids listed in Table 1A were separated with buffer S as electrolyte at −10 kV in <12 min. Table 1 shows working, health-related, and pathological concentrations of these acids in urine; working concentrations are either in the pathological range or in a few cases slightly below it, except for oxalic acid, which is above the pathological range. However, the linear response for this acid in the present conditions has been proven in the range of 250-2000 mmol/mol creatinine, and therefore, lower concentrations can be detected. Within-run (n = 6) and between-run migration times (n = 6) with phthalic acid as IS and without an IS on a single day (within-run) and on 6 different days, with six different electrolyte batches and made by three different operators (between-run) were studied. The within-run CV ranged between 0.17% and 0.65% without an IS and between 0.04% and 0.39% with an IS. Because the between-run CV for migration times is higher without an IS (1.49–2.27%) than it is with one (0.06–1.40%), the use of the IS is especially recommended if the calibrator mixture is not run with the batch.

Electropherogram D in Fig. 1 is a diluted (1:3) urine from a healthy volunteer. No interfering peaks appear in healthy urine under the present conditions (buffer S, −10 kV).

Another six related organic acids—pyroglutamic, orotic, xanthurenic, pyruvic, phenylpyruvic and *p*-hydroxyphenylpyruvic, also included in Table 1—were added to those previously separated. Electropherograms B and C in Fig. 1 show overlapping with some of the other

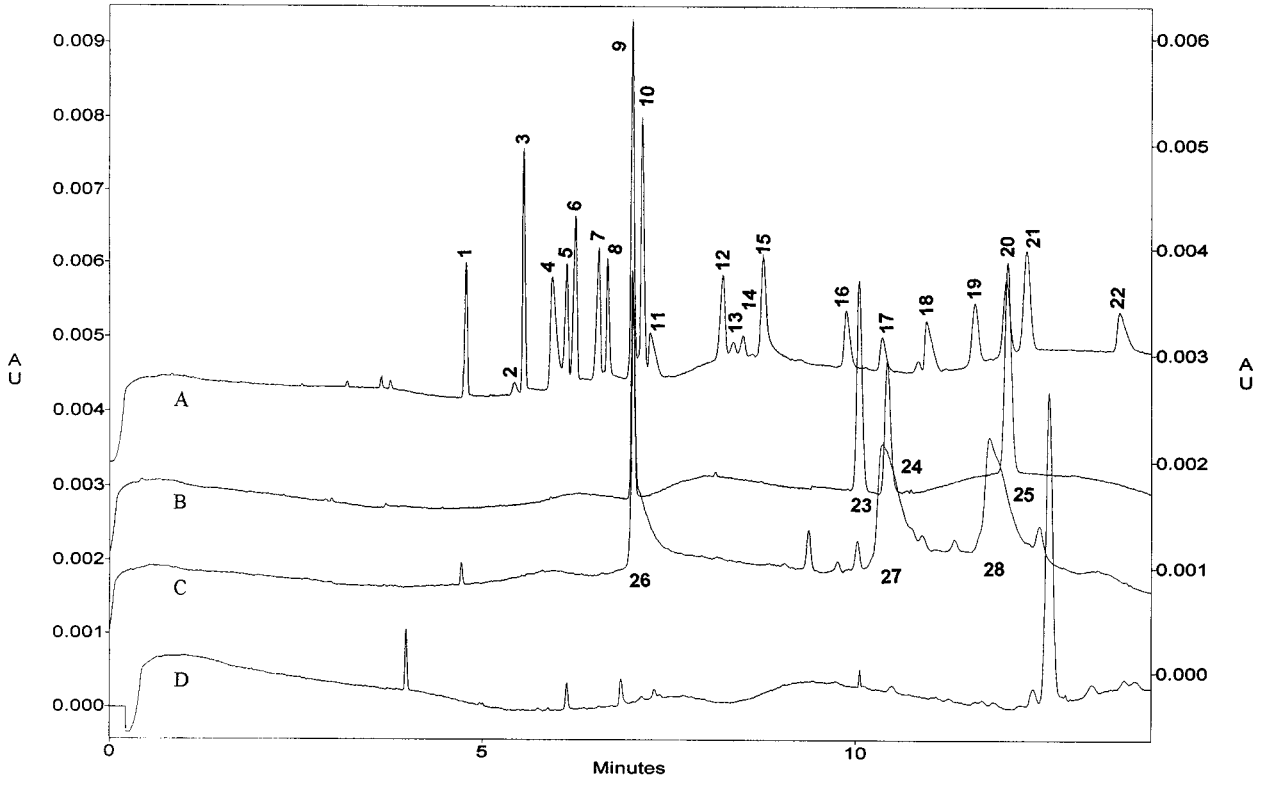

Fig. 1. Electropherograms of calibrators and samples analyzed using buffer S.

Electropherogram A, separation of 22 organic acids by capillary zonal electrophoresis; *electropherograms B* and *C*, new organic acids added. For peak identification, see Table 1A. *Electropherogram D*, healthy diluted (1:3) urine. Applied voltage, −10 kV (see *Materials and Methods).*

Table 1. Organic acid concentrations in healthy and pathological urines.

Peak number	Organic acid	Migration time, min	Working conc, mmol/L	Working conc[a]	Health-related values[a]	Pathological values[a]
A. Buffer S						
Fig. 1. Electropherogram A						
1	Oxalic	4.84	0.80	1000	<100	100–350
2	Oxalacetic	5.48	0.20	250		
3	Fumaric	5.61	0.05	63	<10	3000–4000
4	2-Ketoglutaric	6.00	0.40	500	<150	150–1100
5	Malic	6.19	2.00	2500	<100	
6	Methylmalonic	6.31	2.00	2500	<2	150–15 500
7	Glutaric	6.63	4.00	5000	<10	500–22 000
8	Citric	6.74	0.80	1000	<300	
9	Phthalic (IS)	7.07	0.05	63		
10	*N*-Acetyl-L-aspartic	7.21	0.40	500	<2	1000–7000
11	Glycolic	7.32	4.00	5000	<100	>100
12	Acetoacetic	8.36	0.80	1000	<2	50–20 000
13	Propionic	8.49	1.00	1250		
14	Lactic	8.64	1.00	1250	<100	1000–30 000
15	2-Ketoisovaleric	8.92	0.40	500	<2	300–800
16	3-Hydroxybutyric	10.05	4.00	5000	<100	100–50 000
17	2-Hydroxyisovaleric	10.53	2.00	2500	<2	850–3600
18	4-Methyl-*n*-valeric	11.13	4.00	5000		
19	Phenyllactic	11.82	0.05	63	<2	200–1000
20	Homogentisic	12.24	0.05	63	<2	1000–5000
21	Hippuric	12.53	0.05	63	<300	
22	Aminoadipic	13.81	2.00	2500		
Fig. 1. Electropherogram B						
9	Phthalic (IS)	7.15	0.05	63		
23	Pyroglutamic	10.19	0.50	625	42–115	4000–30 000
24	Orotic	10.57	0.05	63	0–11	30–5600
25	Xanthurenic	12.20	0.05	63		
Fig. 1. Electropherogram C						
26	Pyruvic	7.10	0.80	1000	0–12	50–10 000
9	Phthalic (IS)	7.15	0.05	63		
27	Phenylpyruvic	10.46	0.20	250	0–4	300–1000
28	4-Hydroxyphenylpyruvic	11.90	0.20	250	<2	140–2000
B. Buffer A						
Fig. 2. Electropherogram A						
1	Pyruvic	3.49	0.80			
2	2-Ketocaproic (IS)	4.65	1.00			
3	Orotic	4.83	0.05			
4	Phenylpyruvic	4.93	0.20			
5	Pyroglutamic	5.18	0.50			
6	*N*-Acetyl-L-aspartic	5.26	0.40			
7	Hydroxyphenylpyruvic	5.47	0.20			
8	Xanthurenic	5.98	0.05			
9	Phenyllactic	7.09	0.05			
10	Tropic (IS)[b]	9.58	0.10			
11	Homogentisic	11.88	0.05			

[a] mmol/mol creatinine.
[b] 3-Hydroxy-2-phenylpropanoic acid.

22 compounds. Pyruvic, phenyl pyruvic, and *p*-hydroxyphenyl pyruvic acids with these electrophoretic conditions gave wide peaks, probably because of a tautomeric ketoenolic equilibrium. Pyruvic acid overlaps with phthalic, *N*-acetylaspartic, and glycolic acids; pyroglutamic acid overlaps with 3-hydroxybutyric acid; phenylpyruvic acid

overlaps with orotic and hydroxyisovaleric acids; and *p*-hydroxyphenyl pyruvic and phenyllactic acids overlap with xanthurenic and homogentisic acids. In view of this, if a peak was increased in a suspicious pathological sample in the corresponding migration time, a new buffer system was developed to clearly confirm the compound.

Electropherogram A in Fig. 2 shows the complete separation of the calibrators in Table 1B in a system using buffer A run at −15 kV. Pyruvic acid appeared at 3.49 min in the new buffer; *N*-acetylaspartic appeared at 5.26 min. Glycolic acid did not appear, and phthalic acid interference was easy to eliminate because it was the IS. Pyroglutamic acid appeared at 5.18 min, whereas 3-hydroxybutyric acid did not. Phenylpyruvic acid appeared at 4.93 min and orotic acid at 4.83 min; however, their appearances are very different. Finally, hydroxyisovaleric did not appear; *p*-hydroxyphenyl pyruvic acid appeared at 5.47 min, xanthurenic acid at 5.98 min, phenyllactic acid at 7.09 min, and homogentisic at 11.88 min. To improve precision, two ISs were added: 2-ketocaproic acid (4.65 min) and tropic acid (9.58 min). Within-run (n = 6) imprecision and total between-run imprecision with and without the ISs were compared. Within-run imprecision studies without the IS gave CV values ranging between 0.33% and 1.97%, whereas with relative migration times referred to the closest IS, the CV ranged between 0.10% and 0.75%. The total between-run CV ranged between 1.25% and 4.93% without the IS and between 0.22% and 1.49% with relative migration times referred to the closest IS. Electropherogram B in Fig. 2 belongs to a diluted (1:3) healthy urine analyzed under the same conditions with buffer A and shows no interferences.

SUGGESTED COMPLETE METHOD

First, a daily run of the calibrator mixture is recommended, although when working with an IS, it could be run alone and relative retention times taken as an index. Second, diluted (1:3) and centrifuged samples will be analyzed using buffer S. If any peak with a migration time corresponding to a calibrator increases, a pathology may be suspected; it should be confirmed by co-injecting the presumed compound with the sample or adding the compound to the sample. Third, if one of the compounds corresponding to the retention time of the calibrators listed in Table 1, A and B, increases, a second analysis using buffer A is recommended to confirm the assignment.

ANALYSIS OF URINE SAMPLES

Organic acidurias, although clinically important, are not common, and samples are difficult to obtain. It may be that the diagnosis is missed in some cases because the disorders are not screened at birth. Samples were provided by Hospital Virgen del Rocío, Hospital la Macarena,

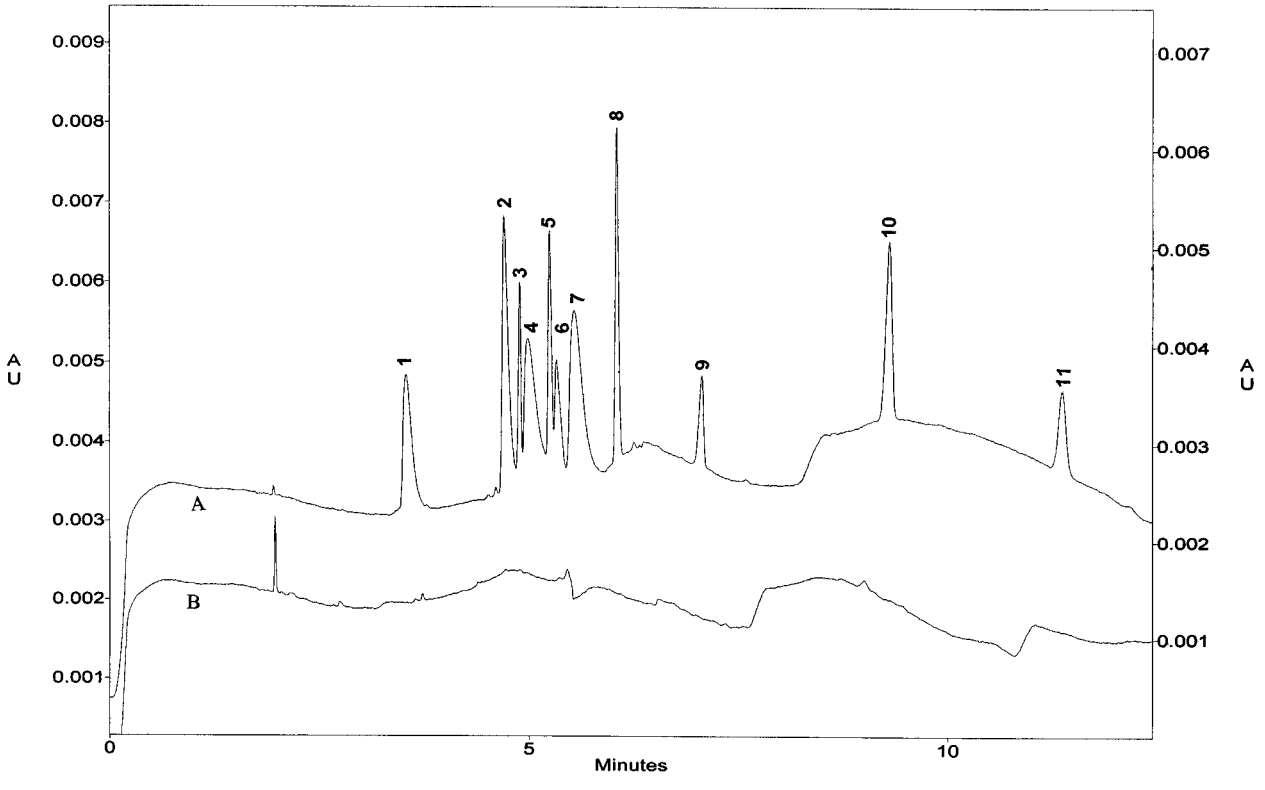

Fig. 2. Electropherograms of peaks that overlapped in buffer S, analyzed using buffer A.

Electropherogram A, separation of the overlapped peaks. For peak identification, see Table 1B. *Electropherogram B*, healthy diluted (1:3) urine. Applied voltage, −15 kV (see *Materials and Methods*).

and Hospital La Paz and were obtained in agreement with the ethics committees of the respective centers. Pathological samples corresponding to methylmalonic and glutaric acidurias were tested following the method described above; the electropherograms are shown in Fig. 3, where healthy urine, pathological samples, and calibrators are included. Pyroglutamic urine was diluted with water using 1 volume of sample and 19 volumes of water (final dilution, 1:20) because of the high concentration of pyroglutamic acid in it. The electropherogram including a healthy urine and the corresponding calibrators is shown in Fig. 4. When a peak related to these diseases appears, it is clearly differentiated from a healthy sample.

Before quantitation, the calibrators and sample linearity were tested; correlation coefficients were found to be >0.99. The concentrations obtained were as follows: methylmalonic acid, 0.011 mol/L (3089 mmol/mol creatinine); pyroglutamic acid, 0.017 mol/L (25 682 mmol/mol creatinine); and glutaric acid, 0.055 mol/L (82 879 mmol/mol creatinine).

The interpretation of organic acid concentrations for diagnostic purposes depends heavily on a pattern of abnormalities because the increase of a single compound may not be diagnostic. Relative amounts of compounds can also be informative.

Some disorders, such as propionic acidemia, methylmalonic acidemia, pyroglutamic acidemia, and glutaric acidemia, can be reliably diagnosed from organic acid excretions because of the consistently high increases of characteristic acids.

Methylmalonic acidemia results from the deficiency of the cobalamin-dependent enzyme methylmalonyl-CoA mutase *(1)*. It is one of the most frequently diagnosed organic acidurias (6 cases in 3 years from 1000 children) *(6)*; chemically the urine of a patient with this disorder is characterized by large amounts of methylmalonic acid, which is almost undetectable in the urine of healthy subjects *(7)*. During a ketotic crisis, the increase of ketone bodies such as 3-hydroxybutyrate is higher than the methylmalonic peak *(8)*. In Fig. 3, the methylmalonic acid peak is clearly increased. Prenatal detection has been accomplished by measurement of methylmalonate in amniotic fluid and maternal urine at midtrimester *(9)*; as in some other inherited metabolic disorders, treatment in the early weeks or months of life is most important *(7)*.

Pyroglutamic acidemia, or 5-oxoprolinuria, is caused by a glutathione synthetase deficiency, an inherited metabolic condition that may show in early infancy as persistent or acute metabolic acidosis associated with chronic hemolytic anemia *(1)*.

The presence or absence of ketonuria associated with metabolic acidosis is the major clinical key to the diagnosis; when metabolic acidosis occurs with an anion gap within reference values and without hyperlacticacidemia or hypoglycemia, pyroglutamic aciduria is rarely diag-

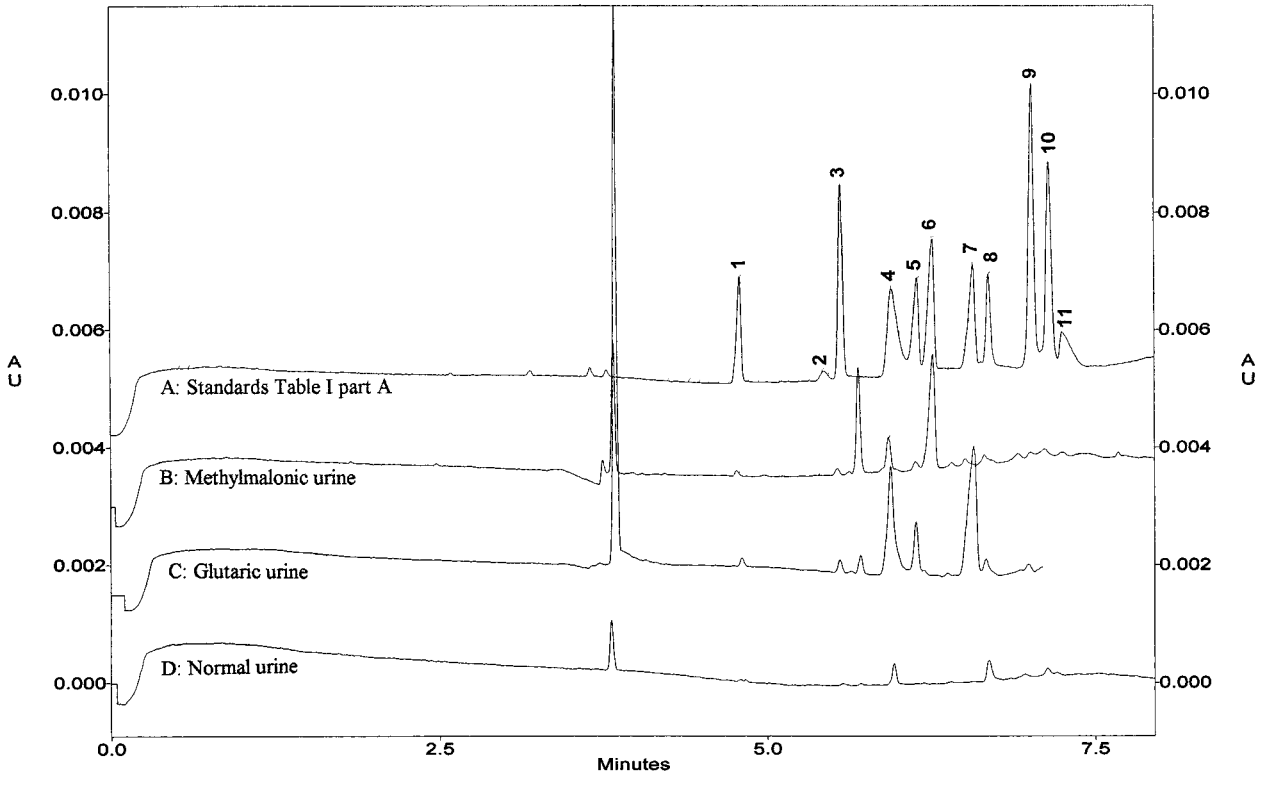

Fig. 3. Electropherograms of urine from patients with methylmalonic or glutaric aciduria compared with healthy urine and calibrators.
Conditions: applied voltage, −10 kV; buffer S (see *Materials and Methods*).

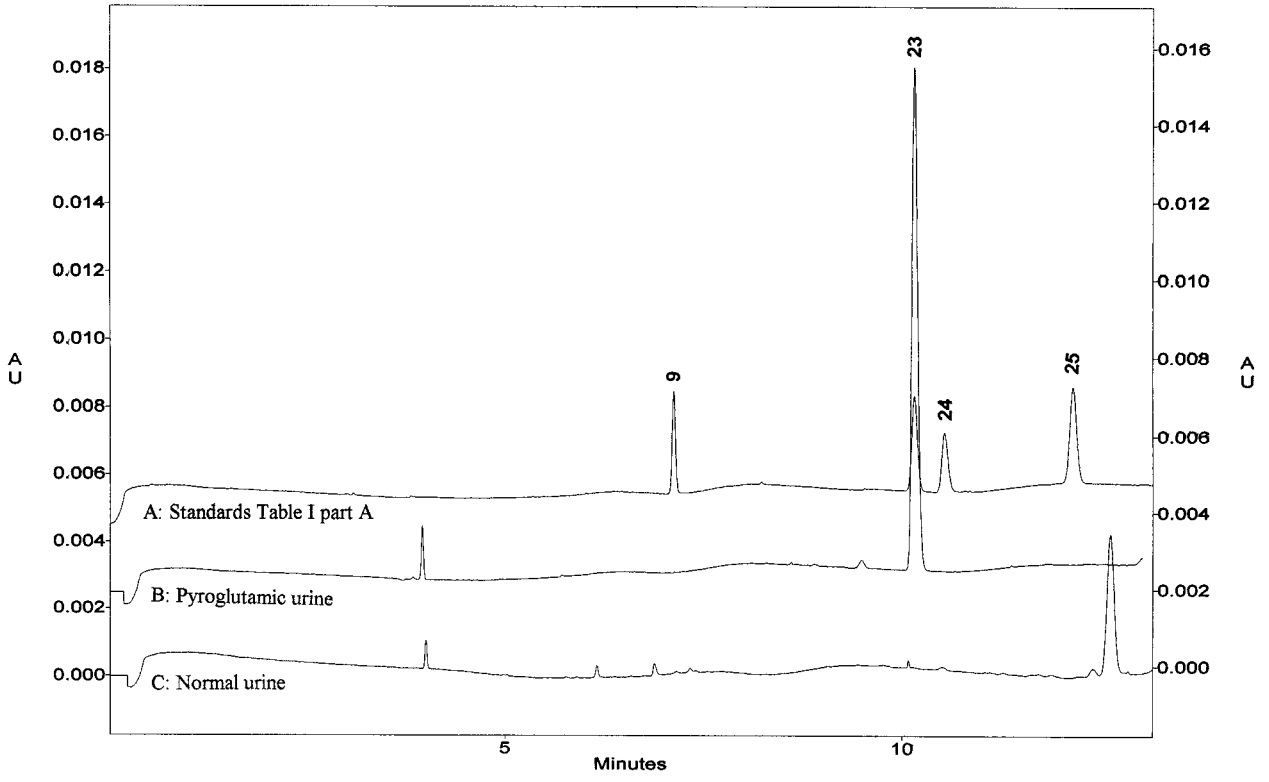

Fig. 4. Electropherograms of urine from a patient with pyroglutamic aciduria compared with healthy urine and calibrators. Conditions: applied voltage, −10 kV; buffer S (see *Materials and Methods*).

nosed (1 case in 3 years from 1000 children) *(6)*, and it may show early in life with constant, isolated metabolic acidosis. If an increased pyroglutamic peak is detected in patient urine, pyroglutamic aciduria may be diagnosed instead of renal tubular acidosis type II *(10)*.

Glutaric acidemia is caused by an isolated deficiency of mitochondrial glutaryl-CoA dehydrogenase (glutaric aciduria type I) or by the deficiency of mitochondrial electron transport flavoprotein or electron transport flavoprotein dehydrogenase *(1)*. Diagnosis is made on the basis of increased glutaric and 3-hydroxyglutaric acids in urine *(10)*. Most infants whose treatment began before the onset of symptoms have developed normally *(11)*, even those with a prenatal diagnosis of glutaric aciduria type I after the discovery of previous cases in the same family *(12)*. Under those circumstances, the interest of early diagnosis is enormous. Fig. 3 shows the marked glutaric peak in a pathological sample, which is >1000-fold higher than in healthy urine *(10)* and is usually out of detection limits.

In reported results on screening of organic acidemias, the incidence ranges between 4% *(7)* and 6.3% *(13)*. However, these reports depend mostly on the selection of patients because, except for classical phenylketonuria, the disorders are not screened at birth. Furthermore, diagnoses could have been missed in some cases because of the severe vital prognosis, unless a specific treatment was immediately instituted, at least for some of them. Moreover, the number of diagnosed inborn errors of metabolism is growing constantly because of the improvement and widespread availability of analytical techniques *(14)*.

Because of the importance of genetic counseling in such diseases, the diagnosis is valuable even in less urgent cases or in postmortem samples *(7)*.

Conclusion

The CE method described as applied to urine is rapid, automated, simple, and inexpensive. It requires only a small volume of urine (50 μL) and no sample preparation. It permits separation, detection, and even identification in <15 min of a wide range of organic acids related to metabolic disorders.

The urgent evaluation of a critically ill newborn is a frequent incident in neonatal intensive care units. Lethargy, coma, vomiting, seizures, and death occur in the first few days of life; thus, the time spent in diagnosis is crucial.

Finally, the proposed method allows the operator to become familiar with the patterns of nonpathological samples and to recognize immediately "true abnormal profiles" in urine of children. It could be a valuable tool in the routine diagnostic system, mainly in newborns, applied to severe diseases that need a specific and early treatment.

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