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Comparison of ion-pair chromatography and capillary zone electrophoresis for the assay of organic acids as markers of abnormal metabolism

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Abstract

The abnormal organic acids in urine are closely related with physiological metabolism. To determinate the low-molecular-mass metabolites in human biological fluids, although there were some previous reports by both of capillary electrophoresis and ion-exchange high-performance liquid chromatography, but it was rarely found by reverse phase of liquid chromatography using ion pair reagent. The objective of this study was aimed to suggest and compare two methods, an additional chromatographic method–ion-pair chromatography (IPC) and a sharp capillary zone electrophoresis (CZE), to determinate organic acids, acting as the abnormal metabolic markers, namely uric acid, orotic acid, pyruvic acid, α-ketoglutaric acid, fumaric acid, and hippuric acid. The proposed method of IPC possessed both the extreme stability for column and the good results of reproducibility, linearity and detection limit. The optimum mobile phase was 22% methanol and 10 mM tetra-*n*-butyl ammonium hydrogen sulfate (pH 4) by gradient elution. As well as the optimum condition of CZE was 5% acetonitrile and 0.5 mM CTAB in phosphate buffer. From the results, CZE showed better recovery and sharp lucid electropherogram. Finally, the two proposed analytical methods were applied to assay human urine with direct and spiked analysis. CZE showed good potency to overcome the sample-to sample variation with standard deviation less than 10%. By comparison results of urinary spiked analysis between IPC and CZE by statistical paired *t*-test, the results were evaluated no significant difference under $P < 0.05$. The quantitative linearity of both methods was fitted in application of clinical biological analysis even with 50-fold dilution.

Keywords: Organic acids as abnormal metabolic markers; Ion-pair chromatography; Capillary zone electrophoresis; Analysis of urine

1. Introduction

In the latest decade, a growing interest has been noted for the determination of organic acids in biological, clinical and food samples.

Some of organic acids found in human serum or urine have known physlogical significance in relation to their concentrations in biological fluids, since they are intermediates formed at various stages of amino acid as indicators of a variety of disease [1]. Organic acids are intermediates in the most important metabolic pathway of carbohydrate, lipids and proteins. Precise measurement of organic acids of abnormal metabolic markers present in biological fluids is essential in order to diagnose certain metabolic disorders [2].

Orotic acid (OA) is an intermediary metabolite in the biosynthesis of pyrimidine nucleotides. The acid is present in healthy adults urine only at trace concentrations in urine of the value of $0.8 \pm 0.3\ \mu mol\ L^{-1}$ [3], however, at the deficiency of enzyme uridine monophosphate synthase, which participates in the synthesis of uridylic acid from orotic acid, the excretion of orotic acid into urine increases (orotic aciduria). It may be about 1000 times higher in urine of patients having orotic acid as a diagnostic metabolite [4]. α-Ketoglutaric acid (KA) is a metabolic marker of α-ketoglutaric aciduria [5]. Fumaric acid (FA) is decomposed from malic acid which will be increased as fumarase deficiency [6]. Hippuric acid (HA) is biosynthesized from glycine, benzoic acid and CoA by enzymes located in the mitochondrial matrix of liver and kidney cells, and is presented at elevated levels in uremia patient's

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urine. This metabolic pathway provides an alternative flux of nitrogen from the usual urea precursors to glycine and it is utilized for the detoxification of aromatic compounds, such as toluene. From the report by Moon et al. [7], it is presented in urine samples from factory workers with ranged from 10 to 1430 ppm [7]. Uric acid (UA) is the final product of purine metabolism is presented in urine and blood and would present at elevated levels in uremia patients urine also [8]. Pyruvic acid (PA) is a metabolite of inborn errors of metabolism, and indicates in pyruvate carbolylase deficiency [9].

To diagnose inborn error of organisms, it is important to use a method enabling for determination of both increased and normal levels of these urinary acids. Their determination has been performed by high-performance liquid chromatography (HPLC) [10], gas chromatography [11–14], ^{1}H nuclear magnetic resonance (NMR) spectroscopy [15], capillary electrophoresis [1,4,16–20], and also by micellar electrokinetic capillary chromatography (MEKC) [21,22]. Although the capillary electrophoresic method had been reported, but they always needed rather complex pretreatment performed due to in acidic medium, such as solid-phase extraction and solid-phase microextraction. The detection limit (0.4–0.6 μmol/L) did rather unsatisfy the trace amount in urine even though combining isotachophoresis (ITP) in the clean up pretreatment and with the aid of diode-array detection (DAD) [19]. Additional, it usually used ion exchange and amino column of HPLC in food analysis [23,24]. The aim of this study was to develop and optimize an additional ion-pair chromatographic method and a capillary zone electrophoresic method in alkali medium with simple and easy pretreatment. Besides, we did the comparison the merits of these two methods.

2. Experimental

2.1. Chemicals

Cetyltrimethylammonium bromide (CTAB) was obtained from TCI (Tokyo, Japan). Acetonitrile and methanol were purchased from J.T. Baker (USA). Hippuric acid, pyruvic acid, trisodium phosphate, disodium phosphate, and tetrabutylammonium hydrogensulfate (TBA) were obtained from E. Merck (Germany). Uric acid and orotic acid were from Aldrich (USA). Then α-ketoglutaric acid and fumaric acid were purchased from Sigma (USA). Deionized water was purified through a purification system which gives a conductivity above 18 MΩ cm. All chemicals used were analytical grade.

2.2. Instrumentation and methodology

2.2.1. Ion-pair chromatography

HPLC apparatus consisted of Perkin-Elmer (Norwalk, CT, USA) 1020 LC plus integrator linked to a model 235C diode-array detector, 250B delivery pump and an injection valve with a 20 μL sample loop. The DAD wavelength of was set at 220 nm. The separations were performed on a Guard-Pak Inserts μBondapak C_{18} in front of a Hypersil BDS C_{18} column (5 μm, 25 cm × 4.6 mm i.d.). The IPC condition was as following: 10.0 mM of TBA–methanol (78:22) for the first 1 min, then increasing to 40% methanol immediately for the following 9 min at flow rate of 1.0 mL/min. The sample was filtered through a 0.45 μm poly(vinylidene difluoride) (PVDF) syringe filter before it was injected into the HPLC system. The identification of the separated compounds was accomplished by co-chromatography with the authentic sample and with the aid of λ_{max} and purity index from DAD and data processing.

2.2.2. Capillary electrophoresis

All of separations were performed with a Spectraphoresis 100 capillary electrophoresis system from Thermo Separation Products TSP (Fremont, CA, USA). The capillary used for separation was 75 μm i.d., 375 μm o.d. and 75 cm length (50 cm to detector). Signal was monitored at 210 nm with a UV–vis SC-100 detector from TSP. Electropherograms were recorded with an SISC-LAB Chromatography Data Station. When capillary tube was first used, it was conditioned with 1 M NaOH for 60 min and then with deionized water for 60 min. Further equilibration was performed with 1 M NaOH for 3 min, deionized water for 3 min and the corresponding running buffer for 3 min. All of the samples including standard solutions were filtered through a 0.45 μm PVDF syringe filter. Standards and samples were injected into the capillary by electrokinetic injection at a fixed time of 4.0 s. The optimized condition was performed at 0.5 mM CTAB and 5% acetonitrile in 10/30 mM phosphate buffer, and applied voltage of −15 kV on fused silica capillary of 75 μm × 75 cm.

2.3. Sample preparation

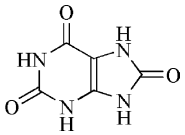
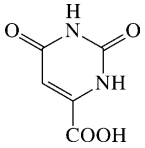
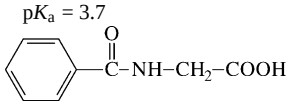
Human urine and blank solution (3 mM trisodium phosphate–methanol, 30:70, pH 11.1) mixed with ratio of 1:1 and then centrifuged at 8000 rpm for 5 min. Pipet 1 mL aliquot and diluted to 25 mL with water for IPC injection. For CZE injection, pipet another 1 mL aliquot and added 2.5 mL of 5 mM CTAB and 2.5 mL of phosphate buffer before diluted to 25 mL. The dilution factor is 50-fold.

3. Results and discussion

3.1. Ion-pair chromatography

The six low-molecular-mass organic acids of abnormal metabolic markers studied were as listed in Table 1. Some of them are hard to dissolve in solvent, such as water, methanol and acetonitrile, etc. So, they needed addition of basic trisodium phosphate in the preparation of stock solution. Organic acids are preferred as anions in basic medium that they could not be separated by simple HPLC because each

Table 1
The structures of six organic acids studied

1. Uric acid (UA) pK_a = 5.83 	4. Pyruvic acid (PA) (2-oxopropionic acid) pK_a = 2.5 $CH_3COCOOH$
2. Orotic acid (OA) pK_a = 1.8 	5. α-Ketoglutaric acid (KA) (2-oxoglutaric acid) pK_{a1} = 3.1, pK_{a2} = 4.4 $HOOCCH_2CH_2COCOOH$
3. Hippuric acid (HA) (*N*-benzoylglycine) pK_a = 3.7 	6. Fumaric acid (FA) pK_{a1} = 3.03, pK_{a2} = 4.44 HOOCCH=CHCOOH

compound may be eluted over solvent without any partition. The application of ion suppression techniques by reversed-phase chromatography in the analysis of ionizable molecules is limited to interesting analytes of weakly basic or acidic functional groups. An attractive alternative to ion exchange and ion suppression analysis of ionic samples is the technique commonly referred to as ion-pair chromatography (IPC). The pH of the eluant is adjusted in order to encourage ionization, for acids pH 7.5 is used and for bases pH 3.5. The chromatographic retention is altered by including an ion pair reagent, a large bulky ion of opposite charge to analyte called counter-ion, in the mobile phase and then which will form a neutral ion-pair with the ionic sample components.

There are three basic models proposed to describe the ion pair mechanism: ion pair, ion exchange and ion interaction [25]. IPC can also be a good choice if the pK_a values of the analytes are very similar and selectivity can be influenced by the choice of the counter ion. From comparison the trial results of pseudoephedrine hydrochloride and TBA, we adopted TBA as the ion-pair reagent in this study. Essentially, we had been trying to addition of TBA in sample solution, that is absence of TBA in mobile phase. But the elution was failed because the analytes were eluted companying with solvent without any partition, that means they were could not be associated with TBA. The pH of TBA solution is around 2.2 without any adjustment. It is over the limitation and may injure the packing of column. So, we adjusted with a solution of 100 mM trisodiumphosphate/100 mM disodium hydrogenphosphate to interesting pH. Accordingly, the pH of 3–6 was studied from considering the pK_a of analytes and the limiting of column. The results were showed in Fig. 1. The analytes may be ionized and associated with TBA from the result in retention. But at pH 6, fumaric and α-ketoglutaric acids were fully overlapped, and orotic, hippuric, and pyruvic

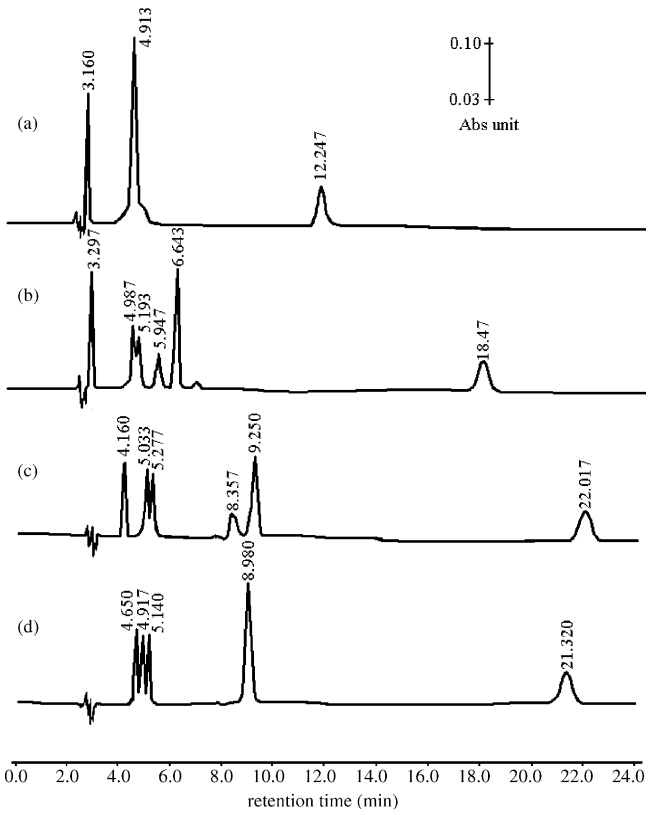


Fig. 1. Effect of pH in IPC. Conditions as is Section 2.2.

acids were partially. On the other hand, it also showed that they could not be associated with TBA and caused to eluting together at pH 3. Consequently, we chose pH 4 for it showed peak symmetry and the better resolution.

The interaction between ion pair and stationary phase was increasing with the concentration of TBA. The range varied from 2.5 to 12.5 mM in 2.5 increments. The influence of various concentrations of TBA is shown in Table 2. Finally, 10.0 mM was chosen as the optimum concentration for a good resolution of orotic and pyruvic acids (R_s = 1.24). We applied gradient elution to shorten the analysis time. The mobile phase used was 10.0 mM of TBA–methanol (78:22) for the first 1 min, then increasing to 40% methanol immediately for the following 9 min. Uric acid, without carboxylic group, is as a neutral type at pH 4, and it was eluted first without any association with TBA. The rest five analytes were eluted with the order of the polarities of ion pairs. Hence, the eluting order was uric, orotic, pyruvic, α-ketoglutaric, fumaric and hippuric at detection wavelength of 220 nm. Furthermore, it increases the reliability of results with the aid of λ_{max} and purity index from DAD and data processing. The chromatogram under optimized condition is shown in Fig. 2. It offers good reproducibility of retention time and peak area (R.S.D. < 0.4 and 2.8%, respectively) with six measurements. The linearity is presented in Table 3. The LODs were obtained by three times of standard deviation by dividing the slope, and the standard deviation was calculated from 20 measurements of five-

Table 2
Effect of TBA on retention and resolution

TBA (mM)	Retention time (min)[a]						
	Uric	Orotic	Pyruvic	α-Ketoglutaric	Fumaric	Hippuric	R_s[b]
12.5	3.23	4.79	5.05	5.88	6.35	20.10	1.21
10.0	3.23	4.83	5.09	5.75	6.20	18.78	1.24
7.5	3.21	4.94	5.17	5.89	6.42	19.42	1.16
5.0	3.19	4.95	5.17	5.85	6.42	18.49	1.04
2.5	3.21	5.12	5.31	6.09	6.86	18.26	0.87

[a] The mobile phase was methanol–TBA (20:80) at flow rate of 1 mL/min, and $n = 3$.
[b] R_s denoted the resolution of orotic acid and pyruvic acid, is calculated by $2[(t_{R2}) - (t_{R1})]/W_1 + W_2$.

Table 3
IPC results of organic acids

Acid	Linearity (ppm)[a]	r	Calibration curve ($\times 10^6$)	LOD (ppm)
Uric	0.1–20.0	0.9982	2.59C + 0.54	0.11
Orotic	0.1–30.0	0.9999	2.91C − 0.12	0.11
Pyruvic	0.4–100.0	0.9997	0.54C + 0.07	0.43
α-Ketoglutaric	1.0–100.0	0.9969	0.46C − 0.08	0.98
Fumaric	0.1–20.0	0.9981	5.18C + 0.47	0.10
Hippuric	0.1–20.0	0.9982	3.38C + 0.42	0.15

[a] The linearity was obtained from 10-level concentrations.

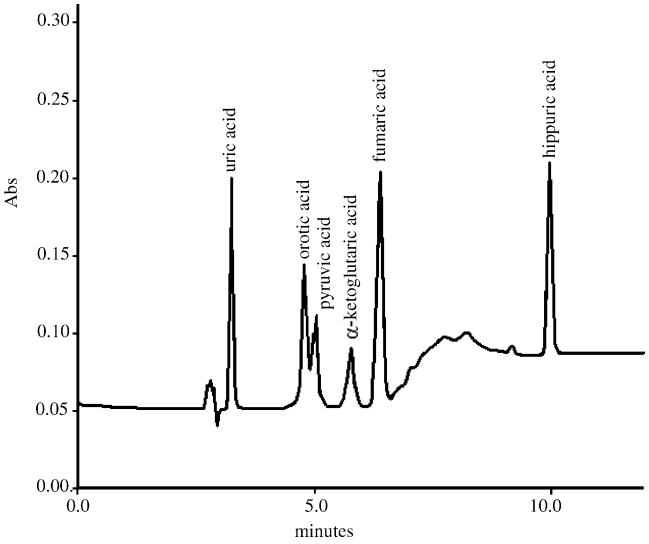


Fig. 2. The optimum chromatogram of organic acids by IPC. Conditions as in Section 2.2.

folds of least-dilution solution. Pyruvic and α-ketoglutaric acids have larger LODs for their rather poor molar absorptivities. But they still could be a suitable determination for abnormal metabolic urine.

3.2. Capillary zone electrophoresis

As already reported papers, the electrolyte employed in the separation of CZE could be optimized with respect to the resolution of abnormal metabolic markers from urine matrix. Considering acid-base properties of analytes and a multicomponent and variable nature of urine matrix it is clear that the CZE separation at a high pH will be faster and be minimized disturbances caused by matrix constituents. This is due to the fact that a number of matrix constituents were precipitated and migrating with the effective mobility close to that of the analyte will be minimized under such alkali medium. Thus, the risk of the overlap of the analyte peak by the matrix constituents will be reduced. Besides, the pK_a of analytes are ranged from 1.8 to 5.8. Thus, all of analytes are anionic under basic electrolyte. Furthermore, we had to select a shorter wavelength because some of analytes are poor detectable in UV region. For this reason above mentioned, borate and phosphate buffer were the appropriate buffer in preliminary test. But an obsession of shifting baseline formed in borate buffer, although it is generally preferred used. Finally, the phosphate buffer was used to achieve this study. On the other hand, we added cationic surfactant, 0.50 mM of CTAB to modify the mobility of electroosmotic flow, and to improve the resolution. To optimize the resolution, we varied the composition of trisodiumphosphate and disodium phosphate at the fixed total concentration of 20 mM. The composition of 5/15 and pH of 11.3 showed the better resolution from the results as shown in Table 4.

Besides the pH, the ionic strength is an important factor that we can use to improve efficiency, resolution and sensitivity of the separation system. Variation of the salt concentration should have identical effects in electropheresis and electroosmosis since they are based on the same principles, both of mobility should be directly proportional to the charge at the surface and to reciprocal of the square root of ionic strength [26]. We continued to vary the concentration of phosphate at the chose ratio of 1:3 by the composition of 5/15, 10/30, and 15/45. The concentration of buffer increased, the mobility of both electrophoresis and electroosmosis reduced. Although

Table 4
Effect of pH on migration time and resolution

Na_3PO_4/Na_2HPO_4 (mM/mM)	pH	Migration time[a] (min)						Resolution	
		Fumaric	α-Ketoglutaric	Orotic	Uric	Pyruvic	Hippuric	R_s[b]	R'_s[c]
15/5	11.8	5.03	5.19	5.27	5.64	5.96	7.72	0.85	3.18
10/10	11.6	4.97	5.14	5.22	5.63	5.89	7.63	0.96	2.62
5/15	11.3	5.24	5.41	5.51	6.05	6.19	8.06	1.14	1.46
3/17	11.0	4.93	5.08	5.18	5.76	5.76	7.33	1.11	0.00

[a] Condition: 5% acetonitrile in 0.5 mM CTAB and applied voltage of −15k V on capillary of 75 μm × 75 cm, and $n = 3$.
[b] R_s denoted the resolution of α-ketoglutaric acid and orotic acid.
[c] R'_s denoted the resolution of uric acid and pyruvic acid.

Table 5
Effect of concentration of phosphate buffer in CZE

Na_3PO_4/Na_2HPO_4 (mM/mM)	Migration time[a] (min)						Resolution	
	Fumaric	α-Ketoglutaric	Orotic	Uric	Pyruvic	Hippuric	R_s[b]	R'_s[c]
5/15	5.24	5.41	5.51	6.05	6.19	8.06	1.14	1.46
10/30	4.76	4.94	5.03	5.51	5.69	7.53	1.05	1.27
15/45	4.18	4.31	4.42	4.82	4.98	6.52	0.99	1.18

[a] The performed condition was 5% acetonitrile in 0.5 mM CTAB, and applied voltage of −15 kV on fused silica capillary of 75 μm × 75 cm, $n = 3$.
[b] R_s denoted the resolution of α-ketoglutaric acid and orotic acid.
[c] R'_s denoted the resolution of uric acid and pyruvic acid.

Table 6
Effect of acetonitrile in CZE

Acetonitrile (%)	Migration time[a] (min)						Resolution	
	Fumaric	α-Ketoglutaric	Orotic	Uric	Pyruvic	Hippuric	R_s[b]	R'_s[c]
2.5	4.74	4.91	4.99	5.42	5.59	7.25	0.95	1.40
5.0	4.76	4.94	5.03	5.51	5.69	7.53	1.05	1.27
7.5	4.86	4.99	5.09	5.55	5.64	7.16	1.01	0.79
10.0	4.75	4.87	4.97	5.40	5.44	6.83	0.99	0.73

[a] Condition: 0.5 mM CTAB and 10/30 mM phosphate buffer, and applied voltage of −15 kV on capillary of 75 μm × 75 cm, $n = 3$.
[b] R_s denoted the resolution of α-ketoglutaric acid and orotic acid.
[c] R'_s denoted the resolution of uric acid and pyruvic acid.

the shorter time was showed at 15/45, but the unacceptable resolution was obtained from the results as shown in Table 5. Consequently, the composition of 10/30 was chosen for its better resolution and shorter time. The poor solubility of analytes was improved by adding of acetonitrile as modifier. But the organic solvent may alter the selectivity, mobility, and increase the solubility of hydrophobic substance [27]. By comparison of the selectivity of methanol and acetonitrile, we chose acetonitrile as organic modifier for the better resolution and peak symmetry. We performed the analysis at 0.5 mM CTAB in 10/30 mM phosphate buffer, and applied voltage of −15 kV on a fused silica capillary of 75 μm × 75 cm, and varied the percentage of acetonitrile, and the results was shown in Table 6. We adopted 5.0% as the optimum amount in this study.

The driving force behind the migration of ions in CZE is the electric field strength applied across the capillary, which is related to the applied voltage (V) over the total capillary length (cm), since both the mobility of electrophoresis and electroosmosis are directly proportional to the electric field strength (V/cm). Larger field strength will result in a shorter analysis time, but the Joule heat production limits the application of high field strength. Therefore, higher efficiency and better resolution would be obtained by working at as highest as possible field strengths. In this work, the capillary length was fixed at 75 cm, and varied the applied voltage of −6, −9, −12, and −15 kV. The optimum separation was achieved at

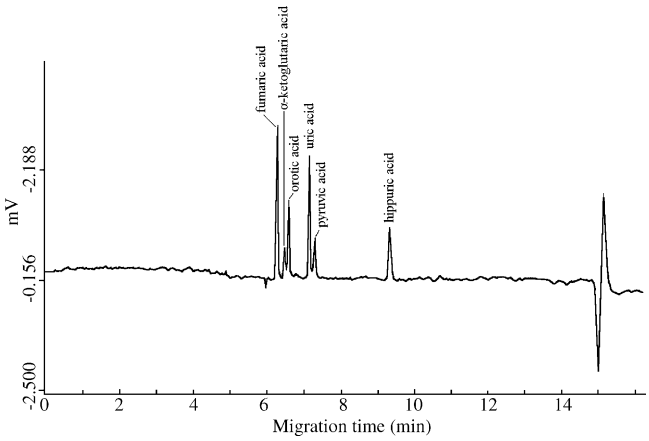


Fig. 3. The optimum electropherogram by CZE. Conditions as is Section 2.2.

Table 7
CZE results of organic acids

Acid	Linearity (ppm)[a]	r	Calibration curve ($\times 10^3$)	R.S.D.[b] (%)	R.S.D.′[c] (%)	LOD (ppm)
Uric	0.7–80.0	0.9975	1.28C + 1.22	1.7	0.6	0.87
Orotic	0.7–80.0	0.9994	0.79C + 0.43	1.3	0.5	0.52
Pyruvic	3.0–70.0	0.9942	0.20C + 0.28	5.4	0.6	2.22
α-Ketoglutaric	5.0–70.0	0.9952	0.12C + 0.26	3.0	0.6	2.39
Fumaric	0.7–80.0	0.9982	1.48C + 1.05	2.4	0.5	0.73
Hippuric	3.0–80.0	0.9996	0.59C − 0.28	2.1	0.9	1.53

[a] The linearity was obtained from 10-level concentrations.
[b] The repeatability of integrated area with five measurements.
[c] The repeatability of migration time with five measurements.

−12 kV, −160 V/cm of electric field strength. The resulting electropherogram under the optimum operation condition is shown in Fig. 3.

The reproducibility of migration time and peak area was determined within-day with five measurements. The results were shown in Table 7. All R.S.D.s were under 0.9 and 5.4%, respectively. The linear range of the analysis was studied by series of injections of standard mixture containing various concentrations of six organic acids. The LOD was obtained with the same method as IPC. The results of linear ranges, correlation coefficient (r), curve, and LOD were all shown in Table 7.

3.3. Assay of human urine

Urine usually contains inorganic constituents (e.g. chloride). This impact of influence is always presence in clinical urinary analysis. The proposed method of IPC and CZE were applied to assay urinary acids of four normal adults by direct and spiked analysis. The electropherograms and chromatograms of sample 2 were indicated in Fig. 4. It showed a

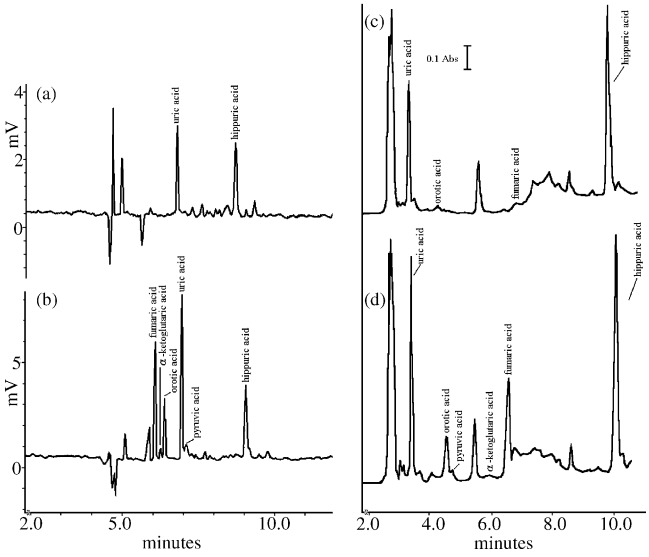


Fig. 4. Electrophoregrams and chromatograms of sample 2. They were performed by CZE (a, b) and IPC (c, d) with direct and spiked analysis. Detailed conditions in Section 2.2.

Table 8
Results of determination of urinary organic acids

Analytical method	Sample	Found (IPC/CZE, ppm)[a]					
		Uric	Orotic	Pyruvic	α-Ketoglutaric	Fumaric	Hippuric
Direct	1	13.0/9.2	0.2/ND[c]	0.4/4.5	1.7/ND	0.1/ND	12.3/3.1
	2	8.9/6.7	0.3/ND	ND/ND	2.3/ND	0.1/ND	16.7/16.6
	3	1.9/1.2	ND/ND	ND/ND	ND/ND	ND/ND	3.9/3.8
	4	7.9/6.7	ND/ND	ND/ND	ND/ND	ND/ND	2.9/3.3
Spiked[b]	1	18.0/22.1	3.6/12.0	1.2/18.5	4.2/13.5	5.1/13.2	17.8/15.1
	2	14.6/23.1	3.6/14.6	1.4/14.7	3.4/15.4	4.7/16.3	21.2/31.6
	3	7.1/16.6	3.6/14.7	1.7/13.3	3.4/15.3	4.8/17.1	8.6/17.9
	4	13.2/21.3	3.6/14.2	2.2/14.9	2.7/12.4	4.6/14.5	8.6/17.0

Sample	Found (IPC/CZE, ppm)[a]					
1	100/86	68/80	16/93	50/90	100/88	110/80
2	113/109	66/97	28/98	22/103	92/109	90/100
3	104/103	72/98	34/89	68/102	96/97	94/94
4	106/97	72/95	44/99	54/83	92/97	114/91
Mean	106/99	70/93	31/95	49/95	95/98	102/91
S.D.	5/10	3/8	12/5	19/10	4/9	12/8

[a] The value was determined by dilution of 50-fold factor with three measurements.
[b] The spiked concentration was 5 ppm for IPC and 15 ppm for CZE.
[c] ND means not detected.

sharp electropherogram, as shown in Fig. 4a and b with simple basify pretreatment and following preparation with phosphate buffer. Although the values of LOD are rather higher than IPC, but its sharp lucid electropherogram is favorable to CZE. The EOF was around 15 min as in standard solution of Fig. 3. And the complex signal around 5 min was speculated as matrices. The results and recovery of real sample analysis were shown in Table 8. Clearly, recoveries of CZE, ranged from 91 ± 8 to 99 ± 10% were rather fine than of HPLC. It showed good ability to overcome the sample-to-sample variation with standard deviation less than 10%. By comparison the contents between IPC and CZE of spiked analysis by statistical paired *t*-test [28], the results were confirmed no significant difference under $P < 0.05$.

4. Conclusions

In summary, the proposed CZE method is suitable as a rapid diagnostic tool for its good recovery, sharp lucid electropherogram and friendly potency to overcome the sample-to-sample variation even its unsignificant value of LOD. And another method, IPC, is still utilizable by spiked analysis for its universality apparatus. And the results of IPC and CZE of spiked analysis were evaluated no significant difference under $P < 0.05$ by statistical paired *t*-test.

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