Research Article

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Bioanalysis

Determination of α-ketoglutaric and pyruvic acids in urine as potential biomarkers for diabetic II and liver cancer

Background: A simple and sensitive hollow fiber-liquid phase microextraction with *in situ* derivatization method was developed for the determination of α-ketoglutaric (α-KG) and pyruvic acids (PA) in small-volume urine samples. 2,4,6-trichloro phenyl hydrazine was used as derivatization agent. **Results:** Under the optimum extraction conditions, enrichment factors of 742 and 400 for α-KG and PA, respectively, were achieved. Calibration curves were linear over the range 1 to 1000 ng/ml ($r^2 \geq 0.998$). Detection and quantitation limits were 0.03 and 0.02, and 0.10 and 0.05 ng/ml for α-KG and PA, respectively. **Conclusion:** The concentrations in diabetic II and liver cancer samples were significantly lower than those from healthy people, showing their potential as biomarkers for these diseases.

Ahmad Makahleh[‡,1], Gazala Mohamed Ben-Hander[‡,1] & Bahruddin Saad[*,1]

[1]School of Chemical Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

*Author for correspondence:
Tel.: +60 4653 2544
Fax.: +60 4656 9869
bahrud@usm.my

[‡]Authors contributed equally

Diabetes mellitus (DM) is a metabolic chronic disease that is characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion and/or insulin action [1]. According to the International Diabetes Federation, currently 382 million people are suffering from DM globally and this number is expected to increase to approximately 592 million by 2035 (~55% increase) [2]. Furthermore, it has been reported that DM is associated with increased risk of cancers [3]. Because of the seriousness of DM and its impact on public health, early prediction and prevention of its occurrence is of great interest.

The abnormal levels of α-keto acids such as pyruvic (PA) and α-ketoglutaric (α-KG) acids, intermediate in the tricarboxylic acid cycle, have been reported to be associated with a variety of metabolic disorders which are the causes of various diseases. For example, the concentration of these acids in serum and urine has been used to investigate the possible diagnosis/treatment of vitamin B_1 deficiency [4], periodontal disease [5], diabetes [6–8], cardiovascular disease [9]. Therefore, the determination of these compounds in body fluids is useful for screening, diagnosis and monitoring of an assortment of pathologies.

The determination of PA and α-KG in biological fluids has been earlier reported [9–20]. As PA and α-KG are nonchromophoric and nonvolatile compounds, LC–MS [9,10,19–22] is the most commonly used technique. High-performance liquid chromatography with ultraviolet (HPLC-UV) [11,23–25] or fluorescence detection [12,13] after derivatization step has also been reported. Although, MS detection and derivatization help to improve sensitivity, high limits of quantitation (LOQ ≥ 200 ng/ml) are still observed [10,19,21]. Alternatively, gas chromatography (GC) with flame ionization detection [14,26] or mass spectrometry [15,16,27] have also been used. Due to their high polarity and low volatility, derivatization step is always mandatory whenever GC analysis is to be performed. To avoid the derivatization step, capillary zone electrophoresis (CE) with UV [17,28], diode array detection (DAD) [29] or capacitively coupled contactless conductivity detection (C^4D) [18] has been also used. However, the lack of sensitivity prevents their analysis of biological fluids.

Different derivatization agents (i.e., ethoxyamine hydrochloride, *o*-phenylenediamine,

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Key terms

α-keto acids: Compounds that have carbonyl and carboxylic groups, are intermediates in several key biological processes such as glycolysis and the tricarboxylic acid cycle.

Hollow-fiber liquid-phase microextraction: Green microextraction technique based on the use of polypropylene fiber for extracting analytes from large donor phase to small acceptor phase.

***In situ* derivatization:** Performing the derivatization together with the extraction.

2,4-dinitrophenylhydrazine, 1,2-diamino-4,5-methylenedioxybenzene, dansylhydrazine, *n*-methyl imidazole, *o*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride and mixture of 1% trimethylchlorosilane in *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide)) have been reported for the analysis of α-keto acids [8,11–13,15,16,20,22,24,27]. A scrutiny through the literature suggests that the use of these derivatization agents is satisfactory, notable disadvantages include the consumption of large amounts of organic solvents [16,27], lengthy evaporation step [15,16,20], unstable derivatives [8,12,24], moisture sensitive reactions [13,20,22], long derivatization time (1–3 h) [15,22,27] and long time of analysis [16,21,22,24]. A lesser known reagent, 2,4,6-trichloro phenyl hydrazine (TCPH) has been used for the off-line derivatization of aldehydes and ketones prior to GC analysis with electron capture [30] or MS detection [31]. Advantageous features of TCPH are the rapid reaction with high stability, cheap and relatively nontoxic.

To date, liquid–liquid extraction (LLE) [16,17,20,26] and solid-phase extraction (SPE) [10,11,19,23,24,27] are the most widely used sample preparation techniques for the determination α-keto acids in biological fluids. However, these techniques suffer numerous drawbacks such as labor-intensive, time consuming, consuming large amounts of sample and toxic organic solvents, involving multisteps procedure (i.e., heating, cooling, centrifugation, evaporation) [32]. To overcome these drawbacks, microextraction techniques such as hollow-fiber liquid-phase microextraction (HF-LPME) was developed as a solvent-minimized pretreatment method that is simple and inexpensive. In addition, the LPME technique often enjoys high analyte enrichment and excellent sample clean-up [33]. The LPME technique can also be performed over a wide pH range compared with the SPE technique [34].

The development of eco-friendly analytical method for the trace determination of these compounds in biological fluids constitutes the main objective of this study. It can be readily anticipated that the high polarity and low detectability suggest that a derivatization step or the use of large volume of sample is necessary. Thus, a three phase HF-LPME with *in situ* derivatization technique using the new derivatization agent (TCPH), followed by the simultaneous HPLC-UV determination of α-KG and PA was attempted. The optimized analytical method was validated and finally applied for the analysis of α-keto acids in small volume of urine samples from normal, diabetic II and liver cancer patients.

Experimental

Chemicals & reagents

Pyruvic acid sodium salt (99.0%), α-ketoglutaric acid (≥99.0%), methanol (HPLC-grade; ≥99.96%), ammonium acetate (98.0%), glacial acetic acid (≥99.8%), hydrochloric acid (37%, w/w) and acetonitrile (HPLC-grade; 99.99%) were purchased from Merck (Darmstadt, Germany). 1-Heptanol (≥99.9%) and 1-octanol (≥99.5%) were purchased from Fluka (Buchs, Switzerland). *n*-decane (99.0%), *n*-tridecane (99.0%) and *n*-octane (97.0%) were from Acros Organics (Geel, Belgium). 2,4,6-trichloro phenyl hydrazine (99.0%), sodium hydroxide (≥98.0%), dihexyl ether (97.0%), *n*-heptane (99.0%) and *n*-hexadecane (99.0%) were purchased from Sigma-Aldrich (MO, USA). Ultrapure water (resistivity, 18.2 MΩ cm^{-1}) was produced by a Milli-Q system (Millipore, MA, USA). TCPH solution (10 mg/ml) was prepared in methanol and was refrigerated (4°C) when not in use.

Instrumentation

Separation of α-KG and PA was performed using a Hitachi LC-6200 intelligent pump (Tokyo, Japan) equipped with a Hewlett-Packard 1050 UV detector (Waldbronn, Germany). Samples were introduced via a Rheodyne 7125 injection valve (CA, USA) with a 5 µl loop. A PowerChrom data acquisition was obtained from eDAQ (Denistone East, Australia) and performed with PowerChrom software (version 2.6.2) for the analysis the chromatographic data. The separation was obtained using a Hypersil GOLD column (250 × 4.6 mm, 5 µm) under a gradient flow rate. The final mobile phase was a mixture of 0.1 M ammonium acetate (pH 4.5, adjusted using 1% acetic acid): acetonitrile (56:44, v/v). The flow rate pr grams were 1.0 ml/min (1–6 min), 1.5 ml/min (7–10 min) and 1.0 ml/min at 11 min. Detection was fixed at 305 nm. Prior to the analysis, the mobile phase was filtered through nylon membrane filter (0.45 µm) from Agilent Technologies (Waldbronn, Germany) and degassed by ultrasonic bath for 15 min. For UV-Vis scanning (200–500 nm), a Waters HPLC system (MA, USA), consisting of Alliance (model 2695) as solvent and sample manager equipped with photodiode array detector (model 2998) and operated with a licensed

empower software (version 2) was used. The extraction was performed using a 25 µl microsyringe with a blunt needle tip (model 702SNR) and it was purchased from Hamilton (NV, USA). A multihotplate stirrer from DAIHAN scientific (Seoul, South Korea) was used for the stirring throughout the extraction process.

Preparation of standard solutions

Stock standard solution (1000 µg/ml) of α-KG and PA mixture was prepared by dissolving the desired amounts in water. Working standard solutions were prepared by suitable dilution of the stock solution in 10 ml volumetric flask.

Urine samples preparation

Urine samples were obtained from 18 volunteers (five healthy, four diabetic and nine liver cancer). All volunteers gave written consent before being enrolled in the study. Prior to the sampling, the volunteers fasted overnight (minimum 6 h). The collected samples were stored immediately in a freezer (-20°C) until use. Urine sample (100 µl) was transferred to a volumetric flask (10 ml) to perform the *in situ* derivatization procedure.

Derivatization & extraction procedure

Standard or urine samples were spiked with 0.1 ml of derivatization solution (10 mg TCPH/1 ml methanol) and 1.0 ml of HCl (1.0 M) as catalyst. The mixture was then diluted in a volumetric flask to 10 ml using water. The solution was transferred to a sample vial (12 ml) containing a magnetic stirring bar (5 × 15 mm). 10 µl of 0.1 M NaOH (acceptor phase) was loaded in a microsyringe and the syringe needle was inserted into a 4 cm length HF segment (Accurel Q3/2 polypropylene hollow fiber membrane (600 µm inner diameter, 200 µm wall thickness and 0.2 µm pore size) that was purchased from Membrana GmbH (Wuppertal, Germany). The HF was bent to a U-shape and the assembly was immersed in 1-octanol for 10 s to impregnate the solvent into the pores wall of the fiber. The fiber was then soaked in water for 5 s in order to wash the extra organic solvent. The HF was placed immediately in the sample vial and the acceptor phase in the syringe was completely injected into the lumen of the HF. The sample solution was agitated at 300 rpm and heated at 70°C for 60 min. At the end of the extraction time, the extract (5 µl) was carefully withdrawn into the syringe and the HF was discarded. Finally, the extract was directly injected into the HPLC system.

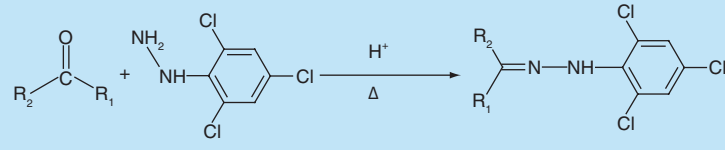
Results & discussion

α-KG and PA are poor chromophore compounds, UV detection is not feasible. Furthermore, direct extraction using the HF-LPME technique is not possible due to their high affinity for water (log P > -0.65) (Figure 1). In order to achieve good detectability and preconcentration, derivatization step is necessary. The derivatization reaction between the carbonyl group of the keto acids and TCPH (schiff base reaction) is shown in Figure 1. The calculated log P of the derivatives suggests significant decrease in their polarity, thus the extraction using the HF-LPME technique should be feasible.

Chromatographic conditions

The separation of the α-keto acids derivatives was investigated using different mobile phase combination and compositions. Methanol, acetonitrile or their combinations were investigated as organic modifier, while water, 0.1% acetic acid or ammonium acetate buffer (pH 3.8–4.5) as aqueous phase component.

Initially, a mobile phase with different ratios of acetonitrile:water, methanol:water and methanol:acetonitrile:water were tested. The peaks of α-KG and PA derivatives were partially overlapped



Analyte	R_1	R_2	Before derivatization		After derivatization	
			Log P	pKa	Log P	pKa
a-KG	C_2H_4COOH	COOH	-1.09	3.1, 4.4	3.54	4.2 ± 0.70
PA	COOH	CH_3	-0.65	2.5	4.07	4.5 ± 0.80

Figure 1. Derivatization reaction between 2,4,6-trichloro phenyl hydrazin and α-keto acids. Log P and pKa values were calculated using ACD/I-lab.
α-KG: α-ketoglutaric acid; PA: Pyruvic acid.

Key term

Enrichment factor: Ratio between the concentrations of analyte in acceptor phase to the donor phase.

when methanol:water or methanol:acetonitrile:water were used. Acetonitrile:water seems to be more promising, but broadening and tailing were observed. In order to improve the peak shape, acetic acid (0.1%) instead of water was investigated. Better separation was indeed achieved as the ratio of acetic acid was increased (>55%). Best peak shapes and shortest run times were obtained when 44:56 (v/v) of acetonitrile:acetic acid (0.1%) was used. However, nonreproducible retention time was observed when the HF-LPME technique was applied. This is due to the use of NaOH as the AP which influences the pH of the mobile phase. In order to overcome this problem, ammonium acetate buffer (0.1 M) was used instead. Different pH (3.8–4.5) of ammonium acetate was investigated. As the pH increases, better baseline and improvement in resolution between analyte peaks and other peaks were observed. pH 4.5 resulted in the best resolution and peak shapes. Higher pH (>4.5) was not considered due to the increase in the retention times of the byproduct peaks. Acetonitrile:0.1 M ammonium acetate (pH = 4.5) (44:56 v/v) was selected. Under these conditions, the retention times for α-KG and PA were 3.2 and 5.4 min, respectively. However, a total run time of 20 min due to the late elution of the derivatization byproduct peaks was required. Therefore, flow rate program was applied to reduce the elution of these peaks. UV-scan (200–500 nm) of the derivatives showed a maximum absorption at 306.7 and 304.3 nm for α-KG and PA, respectively. Thus, 305 nm was chosen for the detection of both analytes. Contrary to the previously reported derivatization reagents, the chosen wavelength is expected to minimize interferences especially from matrix effects. Figure 2 shows a typical chromatogram of the derivatized α-KG and PA under the optimum chromatographic conditions. The corresponding peaks have been identified by performing the derivatization of the blank and each compound individually.

Optimization of extraction conditions

Various parameters (i.e., type of organic solvent, extraction temperature, NaOH concentration (AP), HCl concentration (DP), stirring speed, extraction time and salt addition) that influence the HF-LPME efficiency were studied and optimized.

The choice of organic solvent is important to achieve clean extract with good analyte preconcentration. Type of organic solvent has been investigated using 0.10 M HCl in the DP as catalyst; 0.10 M NaOH (10 µl) as AP; 300 rpm stirring speed; 70°C extraction temperature and 30 min extraction time. Seven organic solvents (dihexyl ether, 1-heptanol, 1-octanol, *n*-octane, *n*-decane, *n*-tridecane and *n*-hexadecane) were examined. 1-octanol showed the highest enrichment factor (EF) for both analytes (Figure 3A). This is due to the unique properties of 1-octanol such as high boiling point (195°C), low solubility in water (0.30 mg/l), fast immobilization in the pores of the fiber (within seconds) and offer good selectivity for the analytes [35]. Thus, 1-octanol was chosen for subsequent experiments.

Low extraction efficiency was observed when carried out at room temperature due to the slow derivatization rate. Therefore, heating was required in order to accelerate the derivatization process and increase the yield of the derivatives [36]. Furthermore, temperature has a considerable effect on the thermodynamics and kinetics of the extraction process. The effect of the extraction temperature (50–80°C) on the extraction efficiency was investigated (Figure 3B). Peak areas were found to increase as the temperature is increased. The highest extraction efficiency was obtained at 70°C and dropped thereafter. The drop in the extraction at higher temperatures (>70°C) is due to the increase in the solubility of 1-octanol in water. Therefore, 70°C was chosen.

The pH of the DP is the most critical factor of the present study. This is due to its influence in providing good derivatization yield and achieves good extraction efficiency. In this study, acid-catalysis is required in order to activate the carbonyl group of the α-keto acids and result in conditions that are favorable for the reaction with TCPH. Furthermore, the estimated pKa values (obtained using ACD/I-lab) of α-KG and PA derivatives were 4.2 ± 0.7 and 4.5 ± 0.8, respectively [37]. Thus, the DP should be sufficiently acidic to ensure the derivatization and extraction take place in a step. The effect of the HCl concentration (0.05–0.20 M) in the DP was investigated. The extraction efficiency was found to increase as the HCl concentration was increased from 0.05 to 0.10 M. An abrupt decrease in extraction was found when the HCl concentration exceeds 0.10 M. This is probably due to the reduction in the derivatization rate which is recommended to be conducted in moderate acidic medium [31].

The pH of the AP should result in complete ionization of the analytes in order to prevent them from back extraction into the organic phase. The AP should be basic in order to promote the dissolution of the acidic analyte. Different concentrations of NaOH (0.05–0.20 M) were studied. The highest extraction efficiency (EE) was obtained when 0.10 M NaOH was used. High concentration of NaOH (>0.10 M) resulted in decrease in the EFs which is due to the possible leaking of NaOH at high concentrations into the DP [38,39]. Therefore, 0.10 M NaOH was selected for further studies.

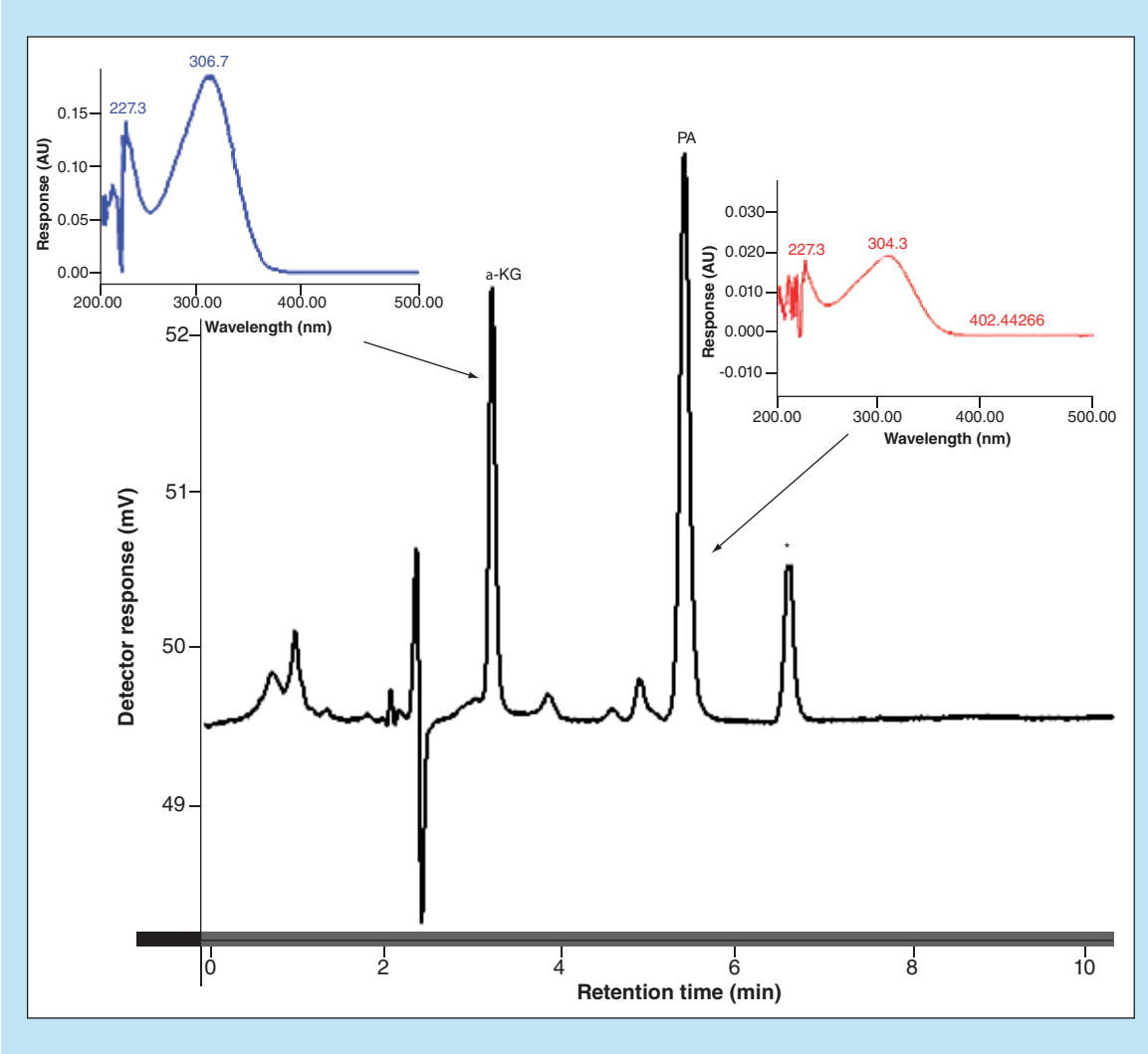


Figure 2. Chromatogram and UV-Vis spectra of α-ketoglutaric acid and pyruvic acid derivatives obtained under the optimum chromatographic conditions. *Indicates a derivatization by product.
α-KG: α-ketoglutaric acid; PA: Pyruvic acid.

The equilibrium between the DP and organic phase can be accelerated by stirring the sample solution. High stirring speeds can enhance the mass transfer and the kinetic rates. However, too high stirring speeds may produce air bubbles on the surface of the HF and the tendency of the solvent to evaporate especially when heating is used [33,39]. The effect of stirring rates (200–900 rpm) on the EE was studied. The highest EE was obtained when the solution was stirred at 300 rpm. Thus, 300 rpm was chosen for further experiments.

Extraction time is another important parameter that affects the EE in HF-LPME techniques. Different extraction times (15–180 min) were investigated. The EF was found to increase as the extraction time was increased up to 120 and 60 min for α-KG and PA, respectively (Figure 3C). Extraction time of 60 min is sufficient for both compounds. Therefore, 60 min was selected. Although, the extraction time was relatively long, using multiple set-ups, many samples can be simultaneously extracted.

Addition of salt can decrease the solubility of analytes in the DP and enhance their partitioning into the organic phase (salting out effect) [39]. Various concentrations of NaCl (0–20%, w/v) were studied. The obtained results showed that the best extraction was achieved when no NaCl was added. It was assumed that the presence of salt may interfere in the derivatization process and reduce the extraction efficiency [33]. Therefore, further experiments were performed without the addition of salt.

The optimum extraction conditions for PA and α-KG were: 1-octanol as organic solvent; 0.10 M HCl in the DP as catalyst; 0.10 M NaOH (10 µl) as AP; 300 rpm stirring speed; 70°C extraction temperature; 60 min extraction time and without addition of salt. Under these extraction conditions, enrichment factors of 742 and 400 for α-KG and PA, respectively, were achieved.

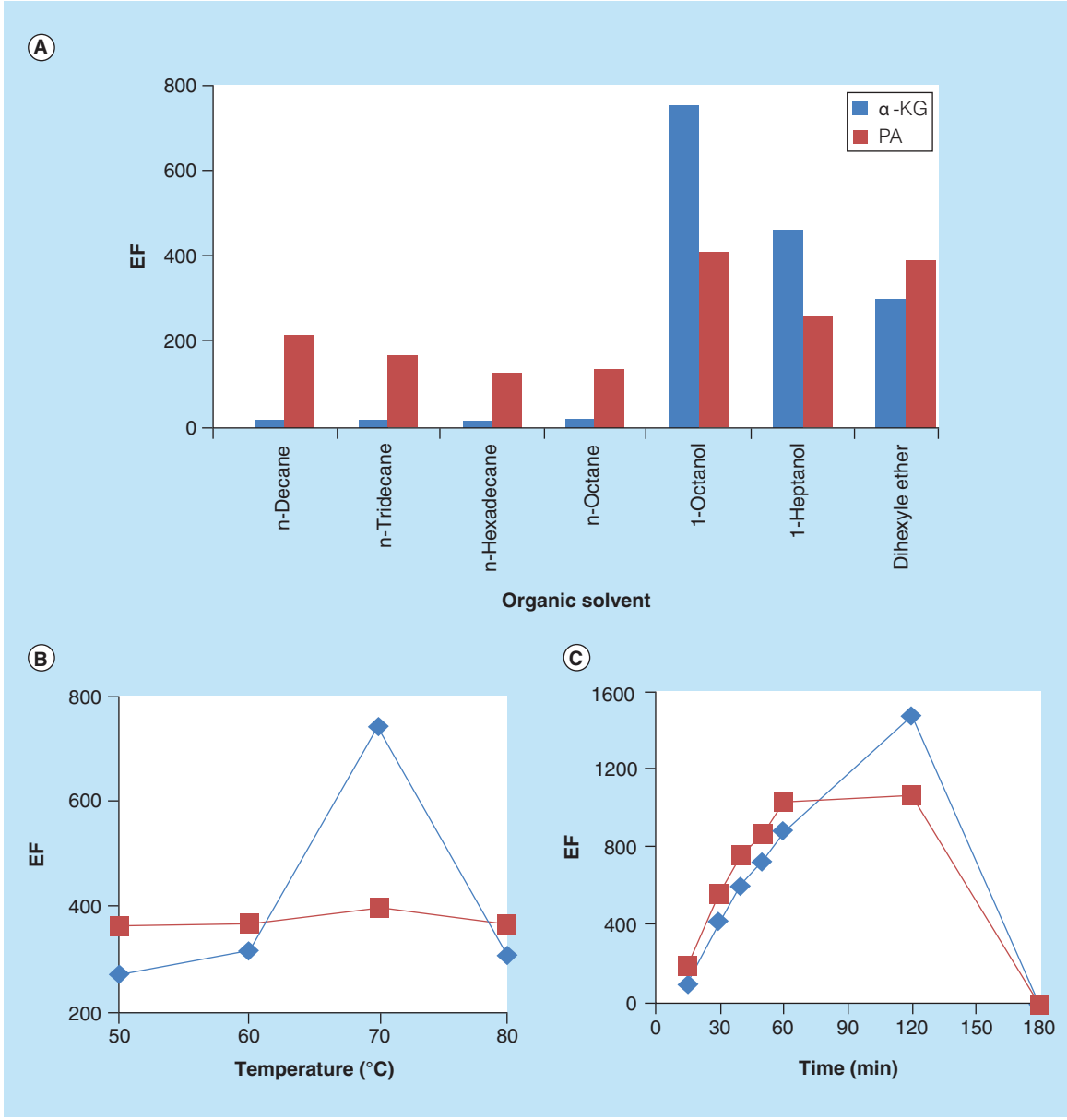


Figure 3. Effect of some parameters on the extraction efficiency (n = 3). (A) Organic solvent using 0.10 M HCl in the DP as catalyst; 0.10 M NaOH (10 µl) as AP; 300 rpm stirring speed; 70°C extraction temperature and 30 min extraction time, **(B)** temperature and **(C)** extraction time.
AP: NaOH concentration; DP: HCl concentration; EF: Enrichment factor; PA: Pyruvic acid.

Validation of the method

Under the optimized conditions, the linearity of the proposed method was investigated over the range 1–1000 ng/ml. Calibration curves were linear over the studied range with regression equations and correlation coefficients of $y = 1.4506\,x - 7.2445$ ($r^2 = 0.998$) and $y = 3.0837\,x - 3.4104$ ($r^2 = 0.999$) for α-KG and PA, respectively. Limits of detection (LOD) and quantitation (LOQ) were calculated at signal-to-noise ratio (S/N) of 3 and 10, respectively. The obtained LODs were 0.03 and 0.02 ng/ml, while the LOQs were 0.10 and 0.05 ng/ml for α-KG and PA, respectively. The obtained results were clearly better in terms of sensitivity over the previously reported methods using SPE with HPLC-UV [11,23,24], solid-liquid extraction with LC–MS/MS [22], SPE with LC–MS/MS [9,21] or SPE with LC–MS [19] and comparable with the reported method using LLE with GC–MS [16] but higher than the reported method using SPE coupled with GC–MS [27]. The relative standard deviations (% RSDs) for six replicate extractions at three different concentration levels (5, 250 and 750 ng/ml) were in the range 11–12% and 8.0–11% for α-KG and PA, respectively (Table 1).

Table 1. Repeatability (%RSD) and recovery in urine sample for α-ketoglutaric acid and pyruvic acid after subjected to the hollow-fiber liquid-phase microextraction method (n = 6).

Analyte	% RSD			%Recovery±SD		
	Concentration (ng/ml)			Spiked level (ng/ml)		
	50	250	750	50	250	750
α-KG	12	11	12	89.8 ± 13	77.0 ± 5.0	93.0 ± 11
PA	11	8.3	8.0	101 ± 15	96.0 ± 7.0	105 ± 12

α-KG: α-ketoglutaric acid; HF-LPME: Hollow-fiber liquid-phase microextraction; PA: Pyruvic acid.

These results indicate the stability of the derivatives that were formed.

Recovery studies were performed by spiking three different concentration levels (50, 250 and 750 ng/ml) to healthy human urine samples. Good recoveries were obtained for α-KG (77.0–93.0%) and PA (96.0–105%), results are shown in Table 1. The obtained results were better than the previous reported recoveries using SPE with HPLC-UV [23,24] and comparable with the reported data using SPE with HPLC-UV [11], direct dilution with LC–MS/MS [10], SPE with LC–MS/MS [9,21] or SPE with LC–MS [19]. Based on these validation results, this method can be applied for the determination of α-KG and PA in human urine samples. A comparison of the proposed method with the previously reported methods is shown in Table 2.

Analysis of urine samples

To demonstrate the clinical applicability of the developed method, urine samples collected from healthy, diabetic II and liver cancer volunteers were tested. Urine samples were chosen as it is easier to be obtained compared with blood samples, and its noninvasive nature readily lends itself as a popular medium for biomarker discoveries [9]. The obtained concentrations in healthy volunteers for α-KG and PA were in the range 41.3–118 µg/ml and 6.44–21.8 µg/ml with average concentrations of 81.8 and 16.2 µg/ml (Table 3), respectively. The concentration of α-KG in all samples was higher than PA which is due to the different metabolism pathways and sources. Generally, no trend or similarity between the samples was observed which due to the different diet, diet behaviors and body metabolism processes. Good reproducibility of

Table 2. Comparison between the proposed hollow-fiber liquid-phase microextraction method with the previously reported methods.

Analyte	Instrument	Detection	Sample preparation	Type of sample	LOD (ng/ml)	Recovery(%)	Ref.
α-KG	HPLC	UV	*In situ* HF-LPME	Urine	0.032	77.0–93.0	Current work
PA					0.015	96.1–105	
PA	HPLC	UV	SPE	Brewed coffee	9000	69.0	[23]
α-KG	HPLC	UV	SPE +Driv	Plasma	13.2	100–104	[11]
α-KG	HPLC	UV	SPE+Driv	Beer	91	68.0–73.0	[24]
PA					55	48.0–50.0	
α-KG	LC	MS/MS	SPE	Urine	0.24	96.9–106	[9]
α-KG	LC	MS/MS	SPE	Fruits, vegetables	0.10 (mg/kg)	81.0–111	[21]
PA					6.4 (mg/kg)	79.0–117	
α-KG	LC	MS	SPE	Plasma, urine	9000	95.6–99.1	[19]
PA	GC	MS	SPE + MAD	Water	0.003	93.0–101	[27]
α-KG	LC	MS/MS	SLE + Driv	Pork meat	0.01(mg/kg)	96.0–120	[22]
PA					0.08 (mg/kg)	87.0–124	
α-KG	GC	MS	Driv+ LLE	Urine, plasma and rat brain tissue	0.03	91.7–95.8	[16]
PA					0.20	90.5–101	
PA	LC	MS/MS	Dilution	Rat urine	200	83.9–89.5	[10]

α-KG: α-ketoglutaric acid; Driv: Derivatization; HF-LPME: Hollow-fiber liquid-phase microextraction; MAD: Microwave-assisted derivatization; PA: Pyruvic acid; UV: Ultraviolet detection.

Table 3. Concentration of α-ketoglutaric acid and pyruvic acid in urine samples obtained from healthy, diabetic II and liver cancer volunteers.

Health status	Volunteer no.	Average ±SD (μg/ml)†		
		α-KG	PA	PA/α-KG ratio
Healthy	1	118 ± 0.41	18.3 ± 0.23	0.15
	2	74.0 ± 0.74	20.6 ± 0.52	0.27
	3	79.5 ± 0.38	6.44 ± 0.10	0.081
	4	95.9 ± 1.0	21.8 ± 0.60	0.22
	5	41.3 ± 0.91	13.7 ± 0.87	0.33
	Range	41.3–118	6.44–21.8	0.081–0.33
	Average	81.8 ± 25	16.2 ± 6.3	0.21
Diabetic II	6	1.27 ± 0.05	0.817 ± 0.04	0.64
	7	19.7 ± 1.0	6.87 ± 0.30	0.35
	8	18.9 ± 0.34	12.2 ± 0.12	0.65
	9	7.92 ± 1.3	2.49 ± 0.20	0.31
	Range	1.27–19.7	0.817–12.2	0.31–0.65
	Average	11.9 ± 8.9	5.59 ± 5.1	0.49
Liver cancer	10	1.41 ± 0.27	0.754 ± 0.07	0.53
	11	6.22 ± 0.89	1.51 ± 0.02	0.24
	12	8.14 ± 1.3	1.75 ± 0.06	0.21
	13	7.28 ± 1.1	2.34 ± 0.08	0.32
	14	0.603 ± 0.02	0.324 ± 0.06	0.53
	15	1.92 ± 0.02	1.35 ± 0.05	0.70
	16	6.29 ± 0.91	8.79 ± 1.0	1.4
	17	8.19 ± 1.2	3.83 ± 0.60	0.46
	18	6.05 ± 0.16	2.51 ± 0.11	0.41
	Range	0.603–8.19	0.324–8.79	0.21–1.4
	Average	5.12 ± 3.0	2.57 ± 2.5	0.54

†Concentration of analyte in 1 ml of urine.
α-KG: α-ketoglutaric acid; PA: Pyruvic acid.

α-keto acids concentrations in urine samples obtained from the same volunteer for different days was observed.

The concentrations in diabetic II and liver cancer volunteers were in the range 1.27–19.7, 0.817–12.2 and 0.603–8.19 and 0.324–8.79 μg/ml for α-KG and PA, respectively. These results were significantly lower than those obtained from healthy volunteers. The significant reduction in concentrations indicates the possibility of using these metabolites as diagnostic biomarkers for diabetes II and liver cancer diseases. Furthermore, the ratios of PA to α-KG for these patients are higher than those of healthy subjects, indicating the disorder in the metabolism process and their association with these diseases. The correlation of α-KG concentration with cardiovascular disease has been previously reported [9]. The reported concentrations of α-KG in cardiovascular patient samples (4.27–27.0 μg/ml) are significantly higher than those obtained from liver cancer patients of the present study, but comparable with diabetic II samples. The reported concentrations for healthy volunteers (18.3–85.8 μg/ml) in that study were comparable to those obtained from the present study [9]. Typical chromatograms of standard, healthy, diabetic II and liver cancer are shown in Figure 4. The chromatograms show clearly that the derivatives were only extracted and no other peaks were observed reflecting the high stability of the derivatives (hydrazones) that were formed. Further clinical investigations are still required to understand detailed relationship between α-keto acids concentrations and these diseases.

Conclusion

For the first time, a HF-LPME technique with *in situ* derivatization followed by HPLC-UV method was

developed for the determination of trace amounts of α-KG and PA in urine samples. The success of the HF-LPME method lies in the marked decrease in polarity of α-keto acids when reacted with TCPH as a new derivatization reagent. The derivatization itself is favorable by the choice of important experimental conditions, especially pH. The proposed method has numerous practical advantages, including the use of small volume of organic solvent, simplicity and high enrichment factors (742 and 400 for α-KG and PA, respectively). The high sensitivity of the method enables the determination of these potential biomarkers in small volumes of urine samples. Significantly lower concentrations of α-keto acids were obtained in diabetic II and liver cancer urine samples in comparison to those from healthy samples, suggesting the possibility of using these metabolites as biomarkers for diabetic II and liver cancer diseases.

Future perspective

The proposed method enables the concentration of α-KG and PA to be readily obtained to establish baseline lends. Fluctuations in their concentrations from those of baseline will signal that an intervention is required. This method offers the prospect of monitoring the health status by profiling the concentrations of α-KG and PA in urine. However, a full long term clinical study is required to prove our finding. This will help in early prediction of diabetic II and liver cancer diseases in order to prevent and/or avoid conditions from worsening specially for overweight people and who has genetic diabetic.

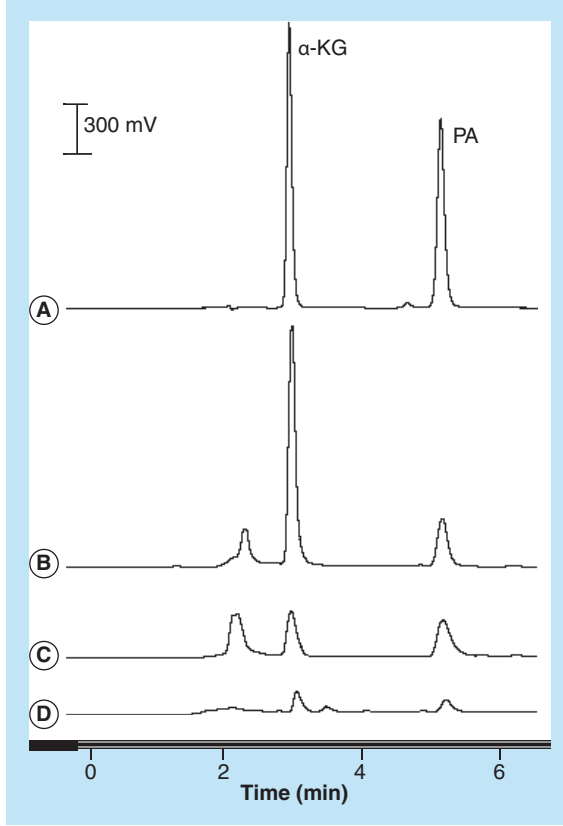


Figure 4. Typical chromatograms of samples subjected to the proposed hollow-fibre liquid phase microextraction method. (A) Working standard (1.0 μg/ml), **(B)** healthy, **(C)** diabetic II and **(D)** liver cancer urine samples.
α-KG: α-ketoglutaric acid; PA: Pyruvic acid.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Method

- HF-LPME with *in situ* derivatization and HPLC-UV method was developed for the first time for the simultaneous determination of α-ketoglutaric acid (α-KG) and pyruvic acid (PA) in healthy, diabetic II and liver cancer urine samples.
- 2,4,6-trichloro phenyl hydrazine was used as derivatization agent.

Results

- The high sensitivity of this method allows the trace determination of α-KG and PA in small volume (100 μl) of urine samples.
- The concentrations of α-KG and PA in diabetic II and liver cancer urine samples were significantly lower than those from healthy people.
- α-KG and PA can be used as potential biomarkers for diabetic II and liver cancer.

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