ISSN 2455-4863 (Online) **www.ijisset.org** *Volume: 5 Issue: 4 | 2019*

Rapid, Simple and Sensitive Derivatization Coupled with Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry for Determination of α-Ketoglutaric Acid and α-Hydroxyglutaric Acid in Rat Plasma

Hongmei Wang, Shuai Li, Sitan Xie, Wuyun Gong, Xiaotong Li, Xin Zhang, Yi Tao*

Pharmacokinetics (DMPK) Unit, Lab-Testing Division, WuXi AppTec Co., Ltd., 288 Fute Zhong Road, Waigaoqiao Free Trade Zone, Pudong New District, Shanghai 200131, China

Abstract: *Two rapid, simple and sensitive methods have been developed for quantification of α-ketoglutaric acid (α-KG) and α-hydroxyglutaric acid (α-HG) in rat plasma. Derivatizations of α-KG and α-HG were performed with 2,4-dinitrophenylhydrazine (DNPH) and benzoyl chloride, respectively. Both of the reactions were processed at room temperature (RT) for 30 min. MeOH:water (v:v, 1:1) was used as surrogate matrix. The α-KG or α-HG derivative was directly analyzed by ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) with different separation protocols. Derivatized α-KG was analyzed using an ACQUITY UPLC BEH C18 (2.1 × 50 mm, 1.7 µm, Waters), while an ACQUITY UPLC HSS T3 column (2.1 × 50 mm, 1.8 µm, Waters) was applied for derivatized α-HG. The chromatographic run time for both methods was 1.8 min/sample. Good linearity (R^2 > 0.99) was obtained for both α-KG and α-HG in the range of 5-5000 ng/mL and 4-6000 ng/mL, respectively. Acceptable precision (CV, ≤11.0%) and accuracy (recovery, ≤112.5%) could be acquired by intra-day and inter-day analysis of duplicated or triplicated quality control samples (QCs) at four different concentrations, which demonstrated the reliability and reproducibility of the current methods. Sprague Dawley (SD) or Wistar Han (HW) rat plasma spiked with known amounts of α-KG (5000 ng/mL) and α-HG (6-4800 ng/mL) showed overall recoveries of 92.8%-102.3% and 95.7%-109.4%, respectively. The methods were rapid, simple and sensitive without extra tedious procedures, such as synthesis of derivatization reagent, liquid-liquid extraction (LLE) or solid phase extraction (SPE). They provided alternative ways to determination of α-KG and α-HG in the biological samples.*

Keywords: *α-Ketoglutaric acid, α-Hydroxyglutaric acid, Derivatization, LC-MS/MS*

1. INTRODUCTION

Both of α-ketoglutaric acid (α-KG) and α-hydroxyglutaric acid (α-HG) are important biomarkers in the human body. α-KG plays significant roles in the Krebs cycle of the organism. It is important for amino acid synthesis as the precursor of glutamine and glutamate, and it participates in several cellular metabolic pathways [1-4]. α-KG is also important in the immune metabolism [5-7], and it could probably extend lifespan [8]. α-Hydroxyglutaric acidurias is characterized with increased concentration of α-hydroxyglutaric acid (α-HG) (D-α-HG and L-α-HG) in the physiological fluids, e.g., urine, serum and CSF [9-11]. In addition, α-HG may be used as indicator of IDH1/2-mutated neoplastic disorders [12-16].

Different analytical techniques have been applied for quantitative determination of α-KG, such as gas chromatography-mass spectrometer (GC-MS) [17-21], and high performance liquid chromatography (HPLC) coupled with different detection techniques, such as ultraviolet detection (UV) [22-23], fluorescence-based detection (FL) [24], and mass spectrometry-based detection (MS) [18, 25-32]. Among all these methods, techniques coupled with MS detection were considered as the most selective and sensitivity way, due to the analyte's structural information provided by the patterns of ion fragmentation. Derivatization was mostly required for GC-MS. However, it was reported that unstable components could be generated during derivatization and resulted in interference peaks [33]. For the reversed phase LC (RPLC), the citric acid cycle acids had poor retention resulting in unfavorable chromatographic behavior, so chemical derivatization [22-24, 29, 32, 34] was usually employed. But these methods either had tedious derivatization [23, 24, 29, 32], or required extra sample preparation steps, such as evaporation and reconstitution [32, 34], liquid-liquid (LLE) [34] or solid phase extraction (SPE) [23, 35], which were impractical for a high-throughput setup. Hydrophilic interaction liquid chromatography (HILIC) coupled to negative mass spectroscopy could be utilized for detection of the organic acid without derivatization [18, 28], but its long re-equilibration time (~4 fold column volumes to equilibrate compared with RPLC) and sensitivity became challenges of this method. In the previous studies, benzofurazan and dansylhydrazine compounds were reported as derivatization reagents for carbonyl compounds. Benzofurazan compounds, e.g. DAABD-AE (4-[2-(N,N-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole), contained both the hydrophobic moieties and ionizable sites improving the analyte's retention and ionization [29].

But DAABD-AE was needed to be synthesized firstly [29, 36] which was preferably avoided when short timeline was required. Dansylhydrazine (DNSH) was a well-known fluorescence labeling reagent. The DNSH derivatives always showed better sensitivity with FL detection [35]. Most of the previous derivatization with DNSH normally required long reaction time (~60 min) [32] at high temperature (~50 ℃) [35], or needed long LC separation (~25-60 min/sample) [32, 35]. Previous studies of α-HG analysis were mainly focus on enantiomeric determination of D-α-HG and L-α-HG using chiral derivatization (e.g., diacetyl-L-tartaric anhydride (DATAN) and N-(p-toluenesulfonyl)-L-phenylalanyl chloride (TPSP)) or chiral column in order to accurate diagnosis of diseases [37-40]. The methods could achieve good separation [37-40] and highly sensitivity [38]. However, most of the studies had multi-step derivatization procedures and long chromatographic protocols. In most cases the sum of D-α-HG and L-α-HG was adequate to meet clients' requirements in the early drug discovery stage. Nowadays, bioanalysts were under intense pressure in terms of shorter turnaround time and high-throughput, together with the increased sample numbers [41-42]. Therefore, the simple sample preparation and short analysis time became a norm [42] in the environment of a contract research organization (CRO). Rapid, simple and sensitive methods were required for quantification of α-KG and α-HG in the bioanalytical field.

Here, we reported two methods for determination of α-KG and α-HG in rat plasma with 2,4-dinitrophenylhydrazine (DNPH) and benzoyl chloride, respectively. Both reactions were easily to handle, which were performed at RT for 30 min. The derivatives could be directly analyzed with UPLC-MS/MS using different LC elution gradients. The acceptable accuracy and precision suggested the reliable and reproducibility of two methods. The methods avoided tedious sample preparation and specific reaction conditions. They provided alternative ways with rapid turnaround time to quantification of α-KG and α-HG in the bioanalytical samples, which were more suitable for screening stage of drug discovery under a CRO environment.

2. MATERIAL AND METHODS

2.1 Chemicals and Materials

α-Ketoglutaric acid (α-KG), D-α-hydroxyglutaric acid disodium salt (abbreviation of free base form was α-HG), glyburide, acetonitrile, methanol, formic acid, ammonium acetate, sodium tetraborate and benzoyl chloride were bought from Sigma-Aldrich (Sanit Louis, MO, USA). (2*S*)-2-hydroxyglutaric acid disodium salt-$^{13}C_5$ (abbreviation of free base form was ^{13}C-α-HG) was purchased from Toronto Research Chemicals (North York, Canada). 2,4-Dinitrophenylhydrazine (DNPH) was purchased from TCI AMERICA (Portland, Oregon, USA). Water was purified with an ELGA water purification system (ELGA, Bucks, UK). The Sprague Dawley (SD) and Wistar Han (HW) rat blank plasma were purchased from BioreclamationIVT (Baltimore, MD, USA).

2.2 Preparation of solutions

2.2.2 α-KG

α-KG was dissolved in purified water:MeOH (v;v, 1:1) to obtain 1 mg/mL stock solution. CSs of α-KG were prepared by serial dilution of stock solution with purified water:MeOH (v;v, 1:1) to the concentrations: 5 ng/mL, 10 ng/mL, 50 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL, 2500 ng/mL and 5000 ng/mL. QCs were prepared by diluting stock solutions with purified water:MeOH (v;v, 1:1) to four different concentrations: 15 ng/mL, 150 ng/mL, 1500 ng/mL and 4000 ng/mL, representing low, medium and high concentrations of the linearity range. IS solution was obtained by dissolving glyburide in MeOH to the concentration of 250 ng/mL. All the concentrations were calculated in free base forms.

2.2.1 α-HG

α-HG was dissolved in purified water:MeOH (v;v, 1:1) to obtain 0.5 mg/mL stock solution. Calibration standards (CSs) were prepared by serial dilution of stock solution with purified water:MeOH (v;v, 1:1) to the concentrations: 4 ng/mL, 8 ng/mL, 20 ng/mL, 100 ng/mL, 200 ng/mL, 1000 ng/mL, 2000 ng/mL and 6000 ng/mL. Quality control samples (QCs) were obtained by diluting stock solution with purified water:MeOH (v;v, 1:1) to four different concentrations: 6 ng/mL, 80 ng/mL, 1600 ng/mL and 4800 ng/mL, which represented low, medium and high concentrations of the calibration range. ^{13}C-α-HG was dissolved in purified water:MeOH (v;v, 1:1) to obtain a concentration of 4 mg/mL. The 4 mg/mL solution was further diluted with purified water:MeOH (v;v, 1:1) to 500 ng/mL using as the internal standard (IS) solution. All the concentrations were calculated in free base forms.

2.3 Derivatization

2.3.2 Derivatization of α-KG with 2,4-dinitrophenylhydrazine

One-hundred μL of 2500 ng/mL CS was mixed with 400 μL of IS solution (250 ng/mL glyburide in MeOH). After vortexing for 1 min, 100 μL aliquot of the supernatant was transferred into a 96-well plate. Ten μL of formic acid and 100 μL of 2,4-dinitrophenylhydrazine (DNPH) were added. After vortexing, the mixture was shaking incubated at RT for 30 min. Three μL of the supernatant was directly injected into UPLC-MS/MS.

2.3.1 Derivatization of α-HG with benzoyl chloride

Sixty μL of 2000 ng/mL CS was mixed with 240 μL of ACN:Water (v:v, 7:3) containing 500 ng/mL ^{13}C-α-HG

as IS. After vortexing for 1 min, 100 µL aliquots of the supernatant were transferred into a 96-well plate, and 100 µL of the 100 mM sodium tetraborate in water and 200 µL of benzoyl chloride:ACN (v:v, 2:98) were added. After vortexing, the mixture was shaking incubated at room temperature (RT) for 30 min. Finally, 3 µL of the supernatant was injected for UPLC-MS/MS.

2.4 Method validation

2.4.1 Linearity, accuracy and precision

In order to evaluate the linearity and sensitivity of the proposed methods, CSs of α-KG (5-5000 ng/mL) and α-HG (4-6000 ng/mL) were prepared and derivatized according to their respective protocols described in sections 2.2 and 2.3. The peak-area ratio of analyte/IS and the nominal concentration of analyte in the calibration standards were used to construct the calibration plots using linear regression. The limits of detection (LODs) were defined as the concentration with the signal-to-noise ratio greater than 3 (S/N > 3). The precision and accuracy were assessed by the coefficient of variation (CV, %) and accuracy (mean ± SD) of QCs at four different concentrations (see preparation procedures in section 2.2 and 2.3). On the day of analysis, QCs at each concentration were paralleled prepared in triplicates and duplicates for α-KG and α-HG, respectively.

2.4.2 Spike-recovery experiment

2.4.2.2. α-KG

The spike-recovery was assessed by adding known amount of the analytes into rat blank plasma followed by derivatization and extraction according to section 2.3. Sprague Dawley (SD) or Wistar Han (HW) rat blank plasma was spiked with 5000 ng/mL of α-KG. The concentration of α-KG in blank plasma was assessed after 100-fold dilution with water due to its high concentration level. The spiked samples were firstly diluted 100 times with water before derivatization. Both spiked samples and blank plasma were prepared in triplicates. The spike-recovery was calculated by detected analyte concentration/(average concentration of α-KG in rat blank plasma + nominal spiked concentration) × 100%.

2.4.2.1 α-HG

Four different concentration levels were tested, and each concentration level was prepared in triplicates. The concentration level of α-HG in SD rat blank plasma was analyzed in triplicates, and the average value was used for calculation of the spike-recovery. Calculation of spike-recovery was the same as α-KG.

2.4.3 Reproducibility

To test the reproducibility of the assay, the inter-day precision and the accuracy of α-KG (15 ng/mL, 150 ng/mL, 1500 ng/mL and 4000 ng/mL) were performed in duplicated QCs on the two different days, while inter-day precision and the accuracy of α-HG at four different concentration (6 ng/mL, 80 ng/mL, 1600 ng/mL and 4800 ng/mL) were performed in triplicated QCs on the three different days.

2.4.4 Ultra-high-performance liquid chromatography/ tandem mass spectrometry

Analysis of α-KG and α-HG were carried out with a Triple Quad™ 6500+ mass spectrometer (SCIEX, MA, USA) connected to an ACQUITY UPLC system (Waters, MA, USA) via an electrospray ionization (ESI) source. Separation of α-KG was using an ACQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm, Waters), while a 1.8 µm-particle size, 2.1 × 50 mm ACQUITY UPLC HSS T3 (Waters, MA, USA) was applied for α-HG. The chromatographic run time was 1.8 min/sample and the flow rate was set at 0.6 mL/min for both methods. For α-KG, the mobile phase gradient was 5% B for the first 0.6 min, and a linear gradient to 95% B at 1.3 min, held for 0.3 min, and back to 5% B for 0.2 min, where A was 0.1% formic acid in water and B was 0.1% formic acid in acetonitrile. For α-HG, the elution gradient was 100% A for the first 0.2 min, a linear gradient to 60% A at 0.5 min, back to 55% A at 1.2 min, and to 5% A at 1.4 min, held for 0.2 min, and finally returned to 100% A for 0.2 min, where A was acetonitrile:purified water (v:v, 5:95) containing 0.1% formic acid and 2 mM ammonium acetate and B was acetonitrile:purified water (v:v, 95:5) containing 0.1% formic acid and 2 mM ammonium acetate. Column temperature was set at 60 °C and 45 °C for α-KG and α-HG, respectively. For both compounds, the mass spectrometer was negative mode with MRM transition. The ESI settings were: nebulizer gas pressure (55 psi), curtain gas (40 psi), auxiliary gas pressure was (60 psi), source temperature (500 °C). MRM parameters were summarized in Table 1.

Table 1. *Parameters of α-KG, glyburide (IS), α-HG and ^{13}C-α-HG (IS) in the MRM analysis. DP: declustering potential. CE: collision energy.*

Compound	Q1 Mass (m/z)	Q3 Mass (m/z)	Dwell Time (msec)	DP (eV)	CE (eV)	Retention time (min)
α-KG	325.0	181.9	15	-60	-22	1.24
Glyburide (IS)	492.2	170.1	15	-77	-41	1.48
α-HG	251.0	128.9	25	-63	-15	0.87
^{13}C-α-HG (IS)	256.0	133.9	25	-40	-15	0.87

3. RESULTS AND DISCUSSION

3.1 Method optimization

In this study, 2,4-dinitrophenylhydrazine (DNHP) and benzoyl chloride were chosen as derivatization reagents to react with α-KG and α-HG, respectively (Fig. 1). The amount of the derivatization reagents and the reaction time were optimized to acquire high conversion yield. Conversion yield was calculated according to Eq. (1). PA was the abbreviation of the peak-area of the analyte at certain time point under a certain reaction condition, and PA_{max} represented the highest peak-area obtained under the same condition.

$$Conversionyield(\%) = \frac{PA}{PA_{max}} * 100\% \qquad \text{Eq. (1)}$$

Different volume of DNPH (20 µL, 50 µL, 100 µL and 200 µL) was mixing with 100 µL sample supernatant and 10 µL formic acid for α-KG, while benzoyl chloride:ACN (v:v, 2:98) (50 µL, 100 µL, 200 µL and 400 µL) was mixing with 100 µL sample supernatant and 100 µL 100 mmol/L sodium tetraborate in the water for α-HG (Fig. 2(A and B)). The mixtures were incubated at RT for 30 min. The highest conversion yields were found in the samples with the volume ratios of sample supernatant:FA:DNHP (v:v:v, 10:1:10) and sample supernatant:sodium tetraborate:2% benzoyl chloride in ACN (v:v:v, 1:1:2) for derivatized α-KG and α-HG, respectively. For derivatization of α-KG, the reaction time was further optimized. The mixture of sample supernatant:FA:DNHP (v:v:v, 10:1:10) was incubated under RT with different time duration (Fig. 2(C)). The highest conversion yield was found in the samples after incubation at RT for 30 min. Finally, derivatization of α-KG with DNPH (v:v:v, sample supernatant:FA:DNHP, 10:1:10) at RT for 30 min was chosen as final protocol, while incubation of α-HG with the volume ratios sample supernatant:sodium tetraborate:2% benzoyl chloride in ACN (v:v:v, 1:1:2) at RT for 30 min was chosen as the reaction condition.

In general, derivatization with DNHP or benzoyl chloride provided simple ways for determination of α-KG and α-HG. The current two derivatization methods avoided complex preparation steps, such as synthesis of the derivatization reagents, or evaporation and extraction procedure.

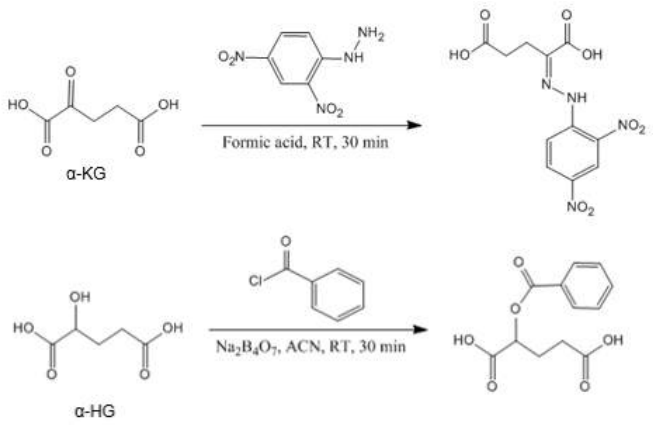

Figure 1. Derivatization of α-KG and α-HG with 2,4-dinitrophenylhydrazine (DNHP) and benzoyl chloride.

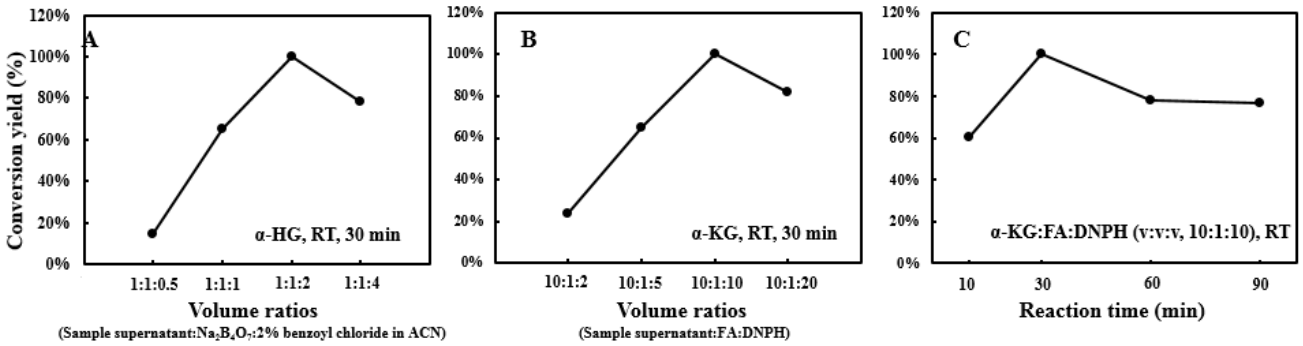

Figure 2. A: Conversion yield of α-HG into corresponding derivative with different amount of benzoyl chloride under RT for 30 min. B: Conversion yield of α-KG into corresponding derivative with different amount of DNPH under RT for 30 min. C: Conversion yield of derivatization of α-KG with DNPH under RT with different reaction time.

3.2 Method validation

The investigated linearity range of α-KG and α-HG were 5-5000 ng/mL and 4-6000 ng/mL, respectively. Good linearity correlations were obtained for both methods with the coefficient of determination (R^2) above 0.99 (Table 2). Chromatograms of limits of detection (LOD)

and upper limit of quantification (ULOQ) of derivatized α-KG and α-HG, and their corresponding internal standards were presented in Fig. 3. The intra-day and inter-day coefficient of variance (CV, %) of different batches of QCs were all below 11.0% and 9.7%, respectively, demonstrating reliability and reproducibility of the methods. For both compounds, the recovery at four different concentrations were within ± 15% of the spiked amount. The validation data suggested that present methods could be applied to accurate quantification of α-KG and α-HG in the surrogate matrix.

Analyte	Linearity Range (ng/mL)	LODs (ng/mL)	R^2	Spiked amount (ng/mL)	Intra-day		Inter-day	
					Precision (CV, %)	Accuracy (recovery %, mean ± SD)	Precision (CV, %)	Accuracy (recovery %, mean ± SD)
α-KG	5-5000	5	0.9966	15	11.0	111.3 ± 12.3	6.6	112.5 ± 7.5
				150	0.0	102.7 ± 0.0	4.0	105.0 ± 4.2
				1500	1.4	101.0 ± 1.4	1.6	101.3 ± 1.6
				4000	5.8	94.4 ± 5.5	3.6	95.0 ± 3.4
α-HG	4-6000	4	0.9972	6	6.6	109.4 ± 7.2	8.0	111.4 ± 8.9
				80	2.1	95.7 ± 2.0	9.7	98.1 ± 9.5
				1600	2.8	98.8 ± 2.7	6.5	105.1 ± 6.8
				4800	2.3	99.6 ± 2.3	4.9	104.9 ± 5.1

Table 2. The linearity range, LOD (limits of detection, S/N > 3), R^2 (coefficient of determination), intra-day and inter-day precision (coefficient of variation, CV, %) and accuracy (recovery, %) of the current methods. The intra-day and inter-day precision and accuracy of α-KG were carried out in duplicated QCs within the same day and on the two different days. For α-HG, the intra-day and inter-day precision and accuracy were performed in triplicated QCs within the same day and on the three different days.

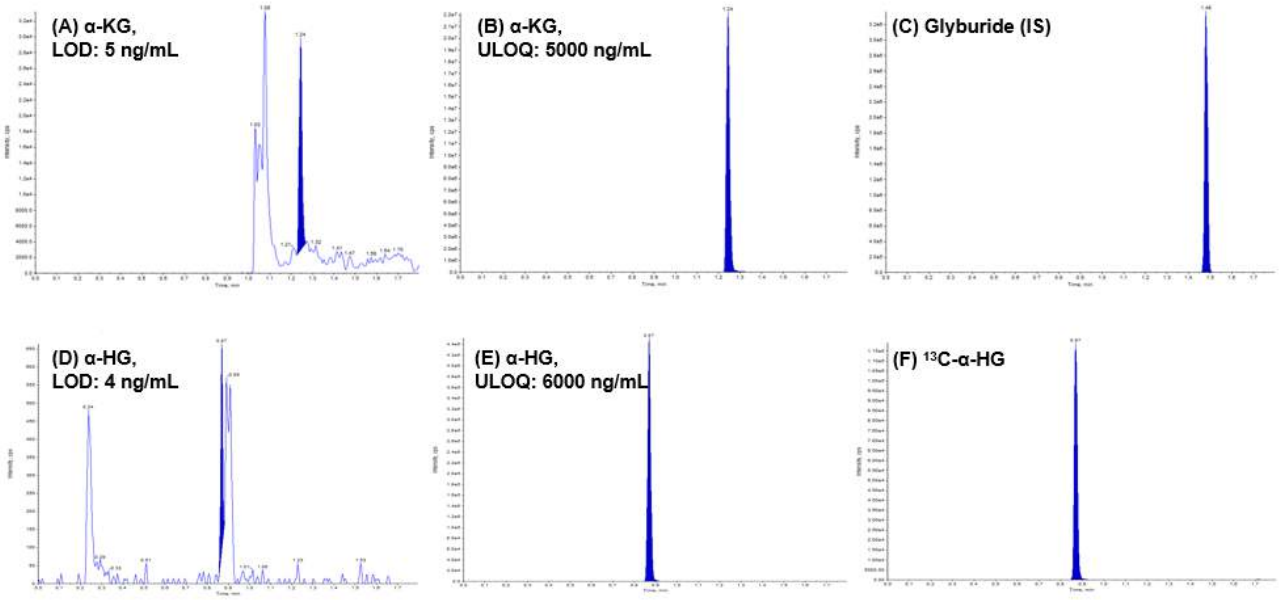

Figure 3. The chromatographs of (A) LOD of derivatized α-KG, (B) ULOQ of derivatized α-KG, (C) Glyburide (IS), (D) LOD of derivatized α-HG, (E) ULOQ of derivatized α-HG and (F) ^{13}C-α-HG.

3.3 Spike-recovery

Spike-recovery experiments were conducted using Sprague Dawley (SD) or Wistar Han (HW) rat blank plasma. α-KG spiked concentration was 5000 ng/ml, while α-HG spiked concentrations were 6, 80, 1600 and 4800 ng/ml. The spike-recovery of α-KG was not investigated for lower concentration due to its high background value in the blank plasma. For α-KG, the precision (CV, %) and the recovery were 4.2% and 92.8% in SD rat plasma, while were 0.3% and 102.3% for HW rat (Table 3). For α-HG, the precision (CV, %) and recovery of the triplicated SD rat plasma at four concentrations were all below 6.6% and 109.4%, respectively. The spike-recovery experiments suggested that current methods could measure α-KG and α-HG added to the normal rat plasma with acceptable and consistent recovery.

Table 3. The precision (coefficient of variation, CV, %) and accuracy (recovery, mean ± SD, %) of the spiked-recovery experiment. The precision and the accuracy at each concentration level were performed in triplicated samples.

Analyte	Original concentration in the plasma (ng/mL)	Spiked amount (ng/mL)	Precision (CV, %)	Accuracy (recovery, mean ± SD, %)
α-KG	9330 (SD rat)	5000	4.2	92.8 ± 3.9
	13800 (HW rat)	5000	0.3	102.3 ± 0.3
α-HG	234 (SD rat)	6	6.6	109.4 ± 7.2
		80	2.1	95.7 ± 2.0
		1600	2.8	98.8 ± 2.7
		4800	2.3	99.6 ± 2.3

4. DISCUSSION

This study presented DNHP and benzoyl chloride derivatization coupled with rapid UPLC-MS/MS (1.8 min/sample) to determination of α-KG and α-HG in the rat plasma, respectively. The acceptable precision (≤11.0%, CV) and accuracy (recovery, ≤112.5%) suggested the reliable and reproducibility of the current methods. The derivatization procedures were easy to handle without any extra steps, e.g., synthesis of derivatization reagents, evaporation and reconstitution procedure or extraction steps. Previous methods without chemical derivatization for α-KG either had relative high LODs [27]; or needed tedious sample preparation, such as SPE purification [23]. Some studies applied hydrophilic interaction liquid chromatography (HILIC) coupled to negative mass spectroscopy, which had poor sensitivity and long re-equilibration time [18, 28]. Determination of α-KG after derivatization with DNSH had been applied [32, 35], but the methods needed SPE step or longer LC separation time (~25-40 min). The method with DAABD-AE derivatization was reported [29]. The reaction was performed at high temperature (60 ℃) for 45 min, and furthermore, DAABA-AE was needed to be synthesized firstly. Studies on quantification of α-HG were mainly focus on chiral separation of the two configurations [37-40]. They needed tedious derivatization or long LC separation time. According to our experience, determination of α-HG, the sum of D-α-HG and L-α-HG, could satisfy clients' requirements in most cases in the early drug discovery stage. In this study, we reported two methods to determination of α-KG and α-HG, respectively. The derivation methods could avoid the rigid reaction condition and tedious sample preparation or reagent synthesis procedure. The analysis time was 1.8 min/sample for both compounds, which was shorter compared to previous methods. The derivatized α-KG and α-HG was eluted at 1.24 min and 0.87 min, respectively. Short derivatization procedures and instrument analysis time were important for method evaluation in the drug discovery stage. If current two methods could be transferred to ADDA-LC-MS/MS platform, the analysis time of one sample could be shorten to half.

5. CONCLUSIONS

Two rapid and sensitive derivatization methods coupled with UPLC-MS/MS were developed **f**or the determination of α-KG and α-HG, respectively. Derivatizations were easy to handle, which were performed with DNPH or benzoyl chloride at room temperature for 30 min incubation. Two rapid 1.8 min UPLC-MS/MS protocols were established, which were shorter than previous studies. These two methods were successfully applied for determination of α-KG and α-HG in SD or HW rats. Acceptable validation results demonstrated the reliable and reproducible of the methods. This study provided an alternative way for the high-throughput analysis of α-KG and α-HG in the biological samples.

ACKNOWLEDGEMENT

We thanked Pharmacokinetics (DMPK) unit of Lab-Testing Division (LTD) in WuXi AppTec Co., Ltd. for financially supporting of this project.

Data Available Statement

The raw data files used to support the findings of this study are available from the authors upon request.

REFERENCES

[1] N. Wu, M. Y, U. G, H. X, Y. Y, D. L, "Alpha-ketoglutarate: physiological functions and applications," *Biomolecules & Therapeutics*, vol. 4, pp. 1-8, 2016.

[2] R. Ledwidge, J.S. Blanchard, "The dual biosynthetic capability of N-acetylornithine aminotransferase in arginine and lysine biosynthesis," *Biochemistry*, vol. 38, pp. 3019-3024, 1999.

[3] U. Hixt, H. Müller, "L-alanyl-glutamine-a glutamine dipeptide for paraenteral nutrition," *Environmental Health Perspectives*, vol. 2, pp. 72–76, 1996.

[4] C. Jones, T. Allan Palmer, R. Griffiths, "Randomized clinical outcome study of critically ill patients given glutamine-supplemented enteral nutrition," *Nutrition*, vol. 15, pp. 108-115, 1999.

[5] S.F. Abcouwer, "Effects of glutamine on immune cells," *Nutrition*, vol. 16, pp. 67-69, 2000.

[6] T.R. Ziegler, N.M. Daignault, "Glutamine regulation of human immune cell function," *Nutrition*, vol. 16, pp. 458-459, 2000.

[7] S.L. Yeh, Y.N. Lai, H.F. Shang, M.T. Lin, W.J. Chen, "Effects of glutamine supplementation on innate immune response in rats with gut-derived sepsis," *British Journal of Nutrition*, vol. 91, pp. 423-430, 2004.

[8] R.M. Chin, X. Fu, M.Y. Pai, L. Vergnes, H. Hwang, G. Deng, S. Diep, B. Lomenick, V.S. Meli, G.C. Monsalve, "The metabolite α-ketoglutarate extends lifespan by inhibiting ATP synthase and TOR," *Nature*, vol. 91, pp. 423-430, 2014.

[9] M. Seijo-Martinez, C. Navarro, M.C. Del Rio, O. Vila, M. Puig, A. Ribes, M. Butron, "L-2-Hydroxyglutaricc aciduria," *Archives of Neurology*, vol. 62, pp. 666-670, 2015.

[10] M. Kranendijk, E.A. Struys, M. Gibson, W.V. Wickenhagen, J.E. Abdenur, J. Buechner, E. Christensen, R.D. De Kremer, A. Errami, P. Gissen, W. Gradowska, E. Hobson, L, Islam, S.H. Korman, T. Kurczynski, B. Maranda, C. Meli, C. Rizzo, C. Sansaricq, F.K. Trefz, R. Webster, C. Jakobs, G.S. Salomons, "Evidence for genetic heterogeneity in D-2-hydroxyglutaric aciduria," *Human Mutation*, vol. 31, pp. 279-283, 2010.

[11] M. Kranendijk, E.A. Struys, G.S. Salomons, M.S. Van Der Knaap, C. Jakobs, "Progress in understanding 2-hydroxyglutaric acidurias," *Journal of Inherited Metabolic Disease,* vol. 35, pp. 571-587, 2012.

[12] P.S. Ward, J. Patel, D.R. Wise, O. Abdel-Wahab, B.D. Bennett, H.A. Coller, J.R. Cross, V.R. Fantin, C.V. Hedvat, A.E. Perl, J.D. Rabinowitz, M.Carroll, S.M. Su, K.A. Sharp, R.L. Levine, C.B. Thompson, "The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting a-ketoglutarate to 2-hydroxyglutarate," *Cancer Cell*, vol. 17, pp. 225-234, 2010.

[13] K.E. Yen, M.A. Bittinger, S.M. Su, V.R. Fantin, "Cancer-associated IDH mutations: biomarker and therapeutic opportunities," *Oncogene*, vol. 29, pp. 6409–6417, 2010.

[14] O.C. Andronesi, G. Kim, E. Gerstner, T. Batchelor, A.A. Tzika, V.R. Fantin, M.G.V. Heiden, A. G. Sorensen, "Detection of 2-hydroxyglutarate in IDH-mutated glioma patients by spectral-editing and 2D correlation magnetic resonance spectroscopy," *Science Translational Medicine*, vol. 4, 116ra4, 2012.

[15] T.A. Juratli, M. Peitzsch, K. Geiger, G. Schackert, G. Eisenhofer, D. Krex, "Accumulation of 2-hydroxyglutarate is not a biomarker for malignant progression in IDH-mutated low-grade gliomas," *Neuro-Oncology*, vol. 15, pp. 682-690, 2013.

[16] E-H Shim, C.B. Livi, D. Rakheja, J. Tan, D. Benson, V. Parekh, E-Y Kho, A.P. Ghosh, R. Kirkman, S. Velu, S. Dutta, B. Chenna, S.L. Rea, R.J. Mishur, Q. Li, T.L. Johnson-Pais, L. Guo, S. Bae, S. Wei, K. Block, S. Sudarshan, "L-2-Hydroxyglutarate: An epigenetic modifier and putative oncometabolite in renal cancer," *Cancer Discovery*, vol. 4, pp. 1290-1298, 2014.

[17] L. Pan, Y. Qiu, T. Chen, J. Lin, Y. Chi, M. Su, A. Zhao, W. Jia, "An optimized procedure for metabonomic analysis of rat liver tissue using gas chromatogrpahy/time-of-flight mass spectrometry," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 52, pp. 589-596, 2010.

[18] S. Yang, M. Sadilek, R.E. Synovec, M.E. Lidstrom, "Liquid chromatography-tandem quadrupole mass spectrometry and comprehensive two-dimensional gas chromatography-time-of-fight mass spectrometry measurement of targeted metabolites of Methylobacterium extorquens AM1 grown on two different carbon sources," *Journal of Chromatography A*, vol. 1216, pp. 3280-3289, 2009.

[19] B.M. Wagner, F. Donnarumma, R. Wintersteiger, W. Windischhofer, H.J. Leis, "Simultaneous quantitative determination of α-ketoglutaric acid and 5-hydroxymethylfurfural in human plasma by gas chromatography–mass spectrometry," *Analytical and Bioanalytical Chemistry*, vol. 396, pp. 2629-2637, 2010.

[20] A.C. Beckstrom, P. Tanya, E.M. Humston, L.R. Snyder, R.E. Synovec, S.E. Juul, "The perinatal transition of the circulating metabolome in a nonhuman primate," *Pediatric Research*, vol. 71, pp. 338–344, 2012.

[21] H. Hur, M.J. Paik, Y. Xuan, D-T. Nguyen, I-H. Ham, J. Yun, Y.K. Cho, G. Lee, S-U. Han, "Quantitative measurement of organic acids in tissues from gastric cancer patients indicates increased glucose metabolism in gastric cancer," *PLOS ONE*, vol. 9, e98581, 2014.

[22] M. Lange, M. Malyusz, "Fast method for the simultaneous determination of 2-oxo acids in biological fluids by high-performance liquid chromatography," *Journal of Chromatography B*, vol. 662, pp. 97-102, 1994.

[23] H. Tsuchiya, I. Hashizume, T. Tokunaga, M. Tatsumi, N. Takagi, T. Hayashi, "High-performance liquid chromatography of α-keto acids in human saliva," *Archives of Oral Biology*, vol. 28, pp. 989-992, 1983.

[24] H. Nohta, J. Sonoda, H. Yoshida, H. Satozono, J. Ishida, M. Yamaguchi, "Liquid chromatographic determination of dicarboxylic acids based on intramolecular excimer-forming fluorescence derivatization," *Journal of Chromatography A*, vol. 1010, pp. 37-44, 2003.

[25] S. Magiera, I. Baranowska, J. Kusa, J. Baranowski, "A liquid chromatography and tandem mass spectrometry method for the determination of

potential biomarkers of cardiovascular disease," *Journal of Chromatography B*, vol. 919-920, pp. 20-29, 2013.

[26] W. McKinnon, C. Pentecost, G.A. Lord, L.G. Forni, J-M. Peron, P.J. Hilton, "Elevation of anions in exercise-induced acidosis: a study by ion-exchange chromatography/mass spectrometry," *Biomedical Chromatography*, vol. 22, pp. 301-305, 2008.

[27] R.I. Dupont Birkler, N.B. Stottrup, S. Hermannson, T.T. Nielsen, N. Gregersen, H.E. Botker, M.F. Andreasen, M. Johannsen, "A UPLC-MS/MS application for profiling of intermediary energy metabolites in microdialysis samples-A method for high-throughput," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 53, pp. 983-990, 2010.

[28] N. Sirboonvorakul, N. Leepipatpiboon, A.M. Dondorp, T. Pouplin, N.J. White, J. Tarning, N. Lindegardh, "Liquid chromatographic-mass spectrometric method for simultaneous determination of small organic acids potentially contributing to acidosis in severe malaria," *Journal of Chromatography B*, vol. 941, pp. 116-122, 2013.

[29] O.Y. AI-Dirbashi, T. Santa, K. AI-Qahtani, M. AI-Amoudi, M.S. Rashed, "Analysis of organic acid markers relevant to inherited metabolic diseases by ultra-performance liquid chromatography/tandem mass spectrometry as benzofurazan derivatives," *Rapid Communications in Mass Spectrometry*, vol. 21, pp. 1984-1990, 2007.

[30] D. Bylund, S.H. Norstrom, S.A. Essen, U.S. Lundstrom, "Analysis of low molecular mass organic acids in natural waters by ion exclusion chromatography tandem mass spectrometry," *Journal of Chromatography A*, vol. 1176, pp. 89-93, 2007.

[31] P. Flores, P. Hellin, J. Fenoll, "Determination of organic acids in fruits and vegetables by liquid chromatography with tandem-mass spectrometry," *Food Chemistry*, vol. 132, pp. 1049-1054, 2012.

[32] F.J. Hidalgo, J.L. Navarro, R.M. Delgado, R. Zamora, "Determination of α-keto acids in pork meat and Iberian ham via tandem mass spectrometry," *Food Chemistry*, vol. 140, pp. 183-188, 2013.

[33] M.G. Horning, E.A. Boucher, A.M. Moss, E.C. Horning, "Gas chromatographic study of derivatives of acids of the Krebs cycle and related compounds," *Analytical Letters*, vol. 1, pp. 713, 1968.

[34] P. Appelblad, E. Ponten, H. Jaegfeldt, T. Backstrom, K. Irgum, "Derivatization of steroids with dansylhydrazine using trifluoromethanesulfonic acid as catalyst," *Analytical Chemistry*, vol. 69, pp. 4905-4911, 1997.

[35] F. Donnarumma, R. Wintersteiger, M. Schober, J. Greilberger, V. Matzi, A. Maier, M. Schwarz, A. Ortner, "Simultaneous quantitation of alpha-ketoglutaric acid and 5-hydroxymethylfurfural in plasma by HPLC with UV and Fluorescence detection," *Analytical Sciences*, vol. 29, pp. 1177-1182, 2013.

[36] Y. Tsukamoto, T. Santa, H. Saimaru, K. Imai, T. Funatsu, "Synthesis of benzofurazan derivatization reagents for carboxylic acids and its application to analysis of fatty acids in rat plasma by high-performance liquid chromatography–electrospray ionization mass spectrometry," *Biomedical Chromatography*, vol. 19, pp. 802-808, 2005.

[37] E.A. Struys, E.E.W. Jansen, N.M. Verhoeven, C. Jakobs, "Measurement of urinary D- and L-2-hudroxyglutarate enantiomers by stable-isotope-dilution liquid chromatography-tandem mass spectrometry after derivatization with diacetyl-L-tartaric anhydride," *Clinical Chemistry*, vol. 50, pp. 1391-1395, 2004.

[38] Q-Y. Cheng, J. Xiong, W. Huang, Q. Ma, W. Ci, Y-Q. Feng, B-F. Yuan, "Sensitive determination of onco-metabolites of D- and L-2-hydroxyglutarate enantiomers by chiral derivatization combined with liquid chromatography/mass spectrometry analysis," *Scientific Reports*, vol. 5, 15217, 2015.

[39] W.M. Oldham, C.B. Clish, Y. Yang, J. Loscalzo, "Hypoxia-mediated increases in L-2-hydroxyglutarate coordinate the metabolic response to reductive stress," *Cell Metabolism*, vol. 22, pp. 1-13, 2015.

[40] W.M. Oldham, J. Loscalzo, "Quantification of 2-hydroxyglutarate enantiomers by liquid chromatography-mass spectrometry," *Bio-protocol*, vol. 6, 2016.

[41] M. Jemal, "High-throughput quantitative bioanalysis by LC/MS/MS," *Biomedical Chromatography*, vol. 14, pp. 422–429, 2000.

[42] R.N. Xu, L. Fan, M.J. Rieser, T.A. El-Shourbagy, "Recent advances in high-throughput quantitative bioanalysis by LC–MS/MS," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 44, pp. 342–355, 2007.