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Quantitative characterization of glutaminolysis in human plasma using liquid chromatography-tandem mass spectrometry

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Abstract

Glutaminolysis is the metabolic pathway that lyses glutamine to glutamate, alanine, citrate, aspartate, and so on. As partially recruiting reaction steps from the tricarboxylic acid (TCA) cycle and the malate-aspartate shuttle, glutaminolysis takes essential place in physiological and pathological situations. We herein developed a sensitive, rapid, and reproducible liquid chromatography-tandem mass spectrometry method to determine the perturbation of glutaminolysis in human plasma by quantifying 13 involved metabolites in a single 20-min run. A pHILIC column with a gradient elution system consisting of acetonitrile-5 mM ammonium acetate was used for separation, while an electrospray ionization source (ESI) operated in negative mode with multiple reaction monitoring was employed for detection. The method was fully validated according to FDA's guidelines, and it generally provided good results in terms of linearity (the correlation coefficient no less than 0.9911 within the range of 0.05–800 μg/mL), intra- and inter-day precision (less than 18.38%) and accuracy (relative standard deviation between 89.24 and 113.4%), with lower limits of quantification between 0.05 and 10 μg/mL. The new analytical approach was successfully applied to analyze the plasma samples from 38 healthy volunteers and 34 patients with type 2 diabetes (T2D). Based on the great sensitivity and comprehensive capacity, the targeted analysis revealed the imperceptible abnormalities in the concentrations of key intermediates, such as iso-citrate and cis-aconitate, thus allowing us to obtain a thorough understanding of glutaminolysis disorder during T2D.

Keywords Glutaminolysis · LC-MS/MS · Isomers · Type 2 diabetes · Human plasma

Introduction

Glutaminolysis consists of a series of biochemical reactions that catabolize glutamine (Gln) to glutamate (Glu), aspartate (Asp), pyruvate (Pyr), lactate (Lac), alanine (Ala), and citrate (Cit) [1, 2]. The degradation of Gln provides a new pillar for energy production by fueling the tricarboxylic acid (TCA) cycle and the malate-aspartate shuttle with its metabolites [3]. Moreover, glutaminolysis is found to be associated with redox balance, mTOR signaling pathway regulation, apoptosis, and autophagy, which can characterize physiological and pathological states in the biological system [4–6]. Hence, the

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Yunfei Hua and Xuping Yang contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00216-019-01626-3) contains supplementary material, which is available to authorized users.

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disorder of glutaminolysis has been found to be closely related to various diseases [7–9]. Untargeted metabolomics studies reported that the levels of metabolites (Cit, Glu, etc.) in glutaminolytic pathway were alerted in the blood and urine samples of type 2 diabetic (T2D) patients [10, 11]. However, the untargeted approach is difficult to accurately characterize the meticulous changes in a specific metabolic pathway, especially involving isomers and low levels of metabolites. The study aims to develop a novel LC-MS/MS method for the simultaneous determination of these metabolites in glutaminolysis in human plasma. The discovered differential metabolites could be applied to diagnosis or targeted therapy for related metabolic diseases in further researches.

The main challenges in simultaneously determining the targeted metabolites (Table 1) in vivo are their high polarity, low molecular weight, and broad concentration range [12]. A number of analytical platforms have been established for the quantification of endogenous metabolites in human plasma, such as gas chromatography-mass spectrometry (GC-MS) [13] and liquid chromatography-mass spectrometry (LC-MS) [14], yet no method has directly accessed all 13 metabolites of interest.

GC-MS is a powerful tool for the analysis of amino acids and organic acids involved in glutaminolysis [15]. However, derivatization, usually conducted in sample preparation of GC-MS, is tedious and time-consuming, even leads to the high complexity of chromatogram by the multi-peak and multi-origination phenomenon [16].

LC-MS technology is considered as a robust measurement of endogenous metabolites for the advantages of flexible separation mechanisms and specific monitoring mode [17]. So far, several LC-MS-based methods for a subset of the metabolites of interest have been reported [18–21]. For example, targeted profiling of 25 polar intracellular metabolites, including malic acid (Mal), fumaric acid (Fum), and Cit, was achieved on a porous graphitic carbon (PGC) column, whereas Lac and Pyr could not be eluted by any mobile phase investigated [19]. Ion pair chromatography (IPC) is another common technique for analyzing these polar molecules. Luo et al. [20] separated 29 negatively charged compounds on a common C18 column by adding tributylammonium acetate (TBAA) as the ion pair modifier. However, the irreversible contamination of MS and column by the ion-pairing reagent limited the application of this method. Additionally, precolumn derivatization was also used to enhance the LC retention and MS detection of endogenous metabolites, such as carboxylates related to TCA cycle [21]. However, the high labor intensity operation of derivatization may lead to inaccurate results.

Here, we present a simple, selective, and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to simultaneously determine 13 metabolites of glutaminolysis in human plasma. To our best knowledge, this is the first report of a comprehensive method that allows these metabolites to be measured in parallel in a single run. The main improvements compared with related state-of-the-art methods were easy sample pre-treatment, good isomer separation, and wide dynamic range. The method was fully validated and successfully applied to comparatively analyze the plasma samples from 38 healthy volunteers and 34 T2D patients. Its high sensitivity and specificity and comprehensive capacity made the present method a good choice to study glutaminolysis in pathological and physiological situations, particularly in energy metabolism–related researches.

Materials and methods

Chemicals and reagents

All standards (Table 1) with purity > 98% were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol (MeOH) and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Deionized water was produced by a Milli-Q system from Millipore (Watford, UK). Formic acid, ammonium formate, ammonium acetate, ammonium bicarbonate, and ammonium hydroxide solution were obtained from Nanjing Chemical Reagent Co. Ltd. (Jiangsu, China).

LC-MS/MS analysis

A Finnigan™ TSQ Quantum Discovery MAXTM LC-MS/MS system (Thermo, USA) was composed of a Finnigan™ Surveyor LC pump, a FinniganTM Surveyor autosampler, and a triple-quadruple TSQ Quantum mass spectrometer. Xcalibur 3.0 software (Thermo, USA) was used to control the instruments and process the data. LC separations were achieved at 35 °C on a SeQuant™ ZIC®-pHILIC column (150 × 4.6 mm, 5 μm, Merck, Germany) with a SHIM-PACK GVP-ODS VanGuard column (2.0 I.D. × 5 mm, 5 μm, Shimadzu, Japan). In addition, ZORBAX SB-Aq column (100 × 2.1 mm, 1.8 μm, Agilent, USA) and XBridge® BEH Amide column (100 × 2.1 mm, 2.5 μm, Waters, USA) were used in our previous attempt. The mobile phase A was acetonitrile and B was water containing 5 mM ammonium acetate. The mobile phases were eluted at 0.5 mL/min following the gradient program: 0–8 min, 85% A → 50% A; 8–13 min, 50% A. A 7-min equilibrium was used before the next injection. The injection volume was 5 μL with partial loop injection, and the thermostatic autosampler tray temperature was kept at 4 °C constantly.

MS/MS detection was accomplished by an electrospray ionization (ESI) source in negative ion mode. The optimized MRM conditions for all analytes are listed in Table 1. Other operating parameters were set as follows: spray voltage 4.5 kV, capillary temperature 275 °C, sheath gas pressure 25

Table 1 List of the structures, mass spectrometer parameters, linearity, and sensitivity for each analyte measured

No.	Analyte	Chemical structure	Molar mass (g/mol)	Precursor ion (m/z)	CE (V)	Production (m/z)	Slope	Intercept	Regression coefficient (r)	Linear range (µg/mL)	LLOQ (µg/mL)
1	Gln		146.1	145.1	13	127.0	18.90	-0.36	0.9973	0.4-200	0.4
2	Glu		147.1	146.0	20	102.0	28.30	0.10	0.9952	0.2-20	0.2
3	Ala		89.1	88.1	12	88.1	65.60	-0.24	0.9991	0.5-100	0.5
4	Asp		133.1	132.0	12	88.0	33.90	-0.18	0.9976	0.2-40	0.2
5	Akg		146.1	145.0	10	101.1	8.15	-1.84	0.9911	1.0-100	1.0
6	Suc		118.1	117.0	13	73.2	9.56	-0.06	0.9972	0.2-10	0.2
7	Fum		116.1	114.9	13	70.9	35.00	0.04	0.9955	0.05-10	0.05
8	Mal		134.1	133.0	13	115.2	13.70	0.03	0.9939	0.5-100	0.5
9	Cis-act		174.1	172.9	15	85.2	10.60	-0.10	0.9957	0.5-200	0.5
10	Pyr		88.1	87.1	6	43.0	75.40	-0.01	0.9962	0.5-200	0.5
11	Lac		90.1	89.1	12	43.0	61.50	7.26	0.9972	8.0-800	8.0
12	Cit		192.1	191.0	12	172.9	363.00	1.87	0.9960	10.0-400	10.0
13	Iso-cit		192.1	191.0	12	172.9	19.40	0.10	0.9953	0.5-20	0.5
14	IS		159.2	158.0	13	140.1	-	-	-	-	-

arbitrary units, auxiliary gas pressure 5 arbitrary units, collision gas (argon) pressure 1.5 mTorr. The scan width and scan time were 0.2 m/z and 0.1 s respectively. High-purity nitrogen served as both nebulizing and drying gas.

Stock and working solution preparation

The standards of Glu, iso-citrate (Iso-cit), Asp, Ala, and Gln were weighed accurately and dissolved in pure water with the concentration of 1.56, 1.56, 3.12, 15.6, and 31.2 mg/mL, respectively, and then stored at 4 °C until utilized. Stock solutions of Fum (1.56 mg/mL), succinic acid (Suc, 1.56 mg/mL),

Mal (15.6 mg/mL), cis-aconitate (Cis-act, 15.6 mg/mL), α-ketoglutaric acid (Akg, 15.6 mg/mL), Pyr (15.6 mg/mL), Cit (31.2 mg/mL), Lac (62.4 mg/mL), and pregabalin (IS, 1 mg/mL) were prepared with methanol and stored in − 20 °C freezer. Combined working solutions were freshly made by serial dilutions of the stocks with MeOH/ACN (50/50, v/v) and stored at 4 °C.

Analyte-free matrix preparation

The analyte-free human plasma for calibration standards and quality control samples (QCs) was prepared by incubating

Table 2 Settings of different conditions for robustness experiment

No.	Flow rate (mL/min)	Additive concentration (mM)	Column temperature (°C)
Condition1	0.5	5	35
Condition2	0.5	4.8	35
Condition3	0.5	5.2	35
Condition4	0.5	5	33
Condition5	0.5	5	37
Condition6	0.48	5	35
Condition7	0.52	5	35

with the activated carbon. Activated carbon was added to the plasma at the ratio of 100 mg/2 mL, then mixed and shaken with 1200 rpm for 6 h at room temperature. After centrifugation at 4 °C, 8000 rpm for 10 min, the supernatant was transferred to another 2.0-mL Eppendorf tube and centrifugated at 4 °C, 8000 rpm for 5 min again. The treated plasma was stored at − 80 °C until use.

Biological sample preparation

To eliminate the potential interferences and extract targeted metabolites from human plasma, 200 μL of MeOH/ACN (50/50, v/v) containing 10 μL IS (8 μg/mL) was added to 50 μL human plasma. The sample was vortexed for 3 min and centrifuged at 16,000 rpm, 4 °C for 10 min. The supernatant liquor was transferred to another tube and centrifuged at 4 °C, 16,000 rpm for 5 min again. Finally, an aliquot of 5 μL supernatant was injected into the LC-MS/MS system for analysis.

Method validation

The validation was conducted according to the FDA draft guidelines on validation of bioanalytical methods with additional experiments for endogenous analytes based on published methods [22]. Validation included lower limit of detection (LLOD), lower limit of quantification (LLOQ), linearity, accuracy, precision, carryover, stability, matrix effect, and extraction recovery.

Calibration curves and QCs

Working solution was spiked with analyte-free plasma to yield a calibration curve. The 6-point calibration curves were constructed by plotting the peak area ratios of all of the metabolites to the IS against the concentrations of the analytes. Based on the statistical analysis of the standard curves, the linear regressions were fitted with $y = ax + b$, and the regression coefficient (r) was calculated by weighting factor $1/x^2$. The

Table 3 Clinical characteristics of 34 T2D patients and 38 healthy volunteers

Characteristics	Normal range	Mean ± SD		
		T2D ($n = 34$)	Control ($n = 38$)	p value[a]
Age (year)		65 ± 11	61 ± 13	0.1025
Gerder (male/female)		24/10	22/16	0.0859
GLU (mmol/L)	4.20–6.40	9.56 ± 2.57	5.14 ± 0.20	0.0000
ALT (IU/L)	9–50	26.65 ± 11.67	15.71 ± 5.74	0.0000
AST (IU/L)	15–40	24.03 ± 7.54	21.11 ± 2.65	0.0380
BUN (mmol/L)	2.90–7.20	5.51 ± 1.09	5.44 ± 1.15	0.8032
Scr (μmol/L)	53–132	82.41 ± 12.42	82.03 ± 11.87	0.8933
TG (mmol/L)	0.45–1.81	2.27 ± 1.84	1.37 ± 0.68	0.0104
TCHO (mmol/L)	2.86–5.98	5.19 ± 1.33	5.14 ± 0.79	0.8581
HDL (mmol/L)	1.07–1.74	1.21 ± 0.25	1.34 ± 0.30	0.0620
LDL (mmol/L)	1.82–3.62	2.81 ± 0.77	2.90 ± 0.61	0.5864

GLU, blood glucose; *ALT*, alanine aminotransferase; *AST*, aspartate aminotransferase; *BUN*, blood urea nitrogen; *Scr*, serum creatinine; *TG*, triglyceride; *TCHO*, total cholesterol; *HDL*, high-density lipoprotein; *LDL*, low-density lipoprotein

[a] Independent samples *t* test was conducted for the differences; χ^2 test was employed when referred to gender

LLOD was calculated at a signal-to-noise ratio of least 3:1. The LLOQ was defined as the lowest concentration on the calibration curve, with an analyte signal-to-noise ratio of 10. The QCs at the concentration of LLOQ, low-quality control (LQC), middle-quality control (MQC), and high-quality control (HQC) were assessed as calibration standards by spiking working solutions at an appropriate concentration.

Accuracy and precision

The inter-day and intra-day accuracy and precision of the method were evaluated by analyzing four concentration levels (LLOQ, LQC, MQC, and HQC), and each level contained five replicates on the same day and three validation days. The accuracy (%) at each concentration was calculated by the percentage ratio of measured concentrations of QC samples to the nominal value according to calibration curves. The precision (%) was expressed by the relative standard deviation (RSD). The acceptance criteria for accuracy are 85–115%,

a

b

while for precisions are no more than 15% deviation from the nominal concentration, except for LLOQ which is acceptable in the range of 80–120% and within 20% deviation, respectively.

Carryover

Carryover was tested by sequentially injecting a blank standard solution MeOH/ACN (50/50, *v/v*) after monitoring an upper limit of quantification (ULOQ) sample. The signal response in the blank standard solution at the retention times of the metabolites and IS should be less than 20% of the mean response of the LLOQ sample.

Stability

Stability of each analyte was tested by comparison of the QC concentrations under different conditions with the freshly prepared sample concentrations. Short-term storage (6 h at room temperature) stability, long-term storage (30 days at − 80 °C) stability, autosampler storage (4 °C for 12 h) stability, and three freeze-thaw cycles stability of QC samples were evaluated by analysis of samples at each concentration level of QCs (LQC, MQC, and HQC). Final QC samples were considered stable if the accuracy was within 85–115% of nominal concentrations and the RSD within 15%.

Matrix effect

Matrix effect was used to evaluate the enhancement and suppression of the ionization and was investigated at three QC levels and obtained by comparing the mean peak area ratio (analyte/IS) of the analytes in six replicates of post-extraction samples with the corresponding neat solution. The matrix effect was accepted if the ratio in the range of 85–115%.

Extraction recovery

Recovery experiment was carried out at three levels of QCs (LQC, MQC, and HQC). Moreover, the extraction recovery was calculated by the following equation: $\mathrm{ER}\% = A_{\mathrm{S\text{-}R}} \; A_{\mathrm{R\text{-}R}} \times 5 \quad 4 \times 100\%$, where $A_{\mathrm{S\text{-}R}}$ is the peak area ratio of each analyte extracted from spiked samples (250-μL aliquots) to IS added in post-extraction samples and $A_{\mathrm{R\text{-}R}}$ is the mean peak ratio of same amount analytes to IS spiked into the post-extraction samples (200-μL aliquots) which represent 100% recovery. Five replicates were included at each QC level.

Robustness

The robustness of a method is a measure of its capacity to remain unaffected by deliberate variations in method parameters, and it indicates reliability during normal usage. A
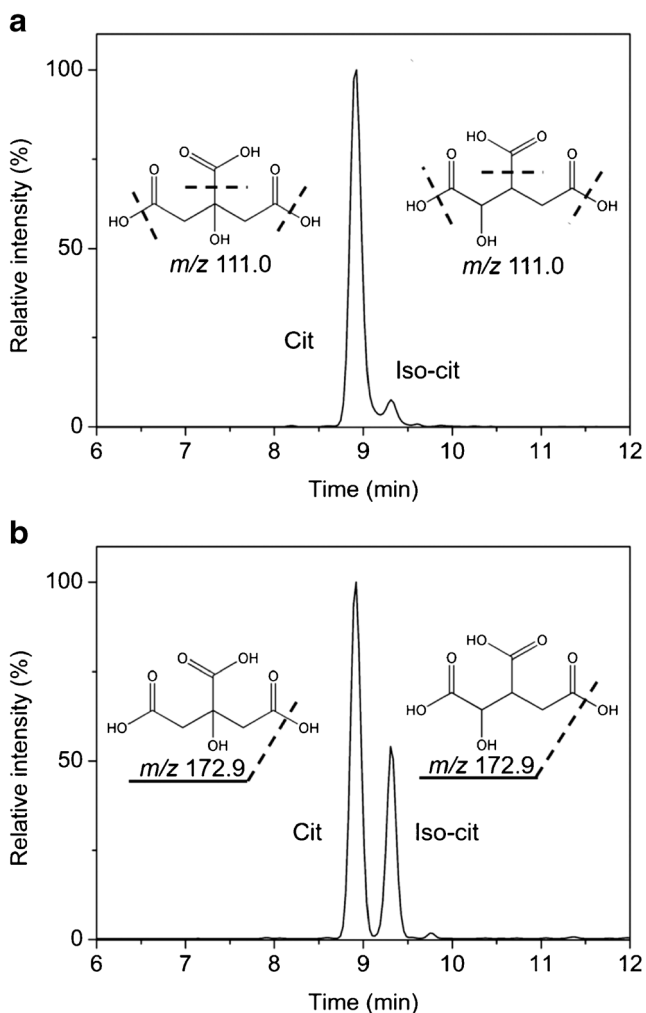
Fig. 1 MRM chromatograms and possible fragmentations of Cit and Iso-cit in human plasma. Selected reaction monitoring transitions are **a** *m/z* 191.0–111.0 and **b** *m/z* 191.0–172.9

Table 4 Intra- and inter-day precisions and accuracies of analytes

Analyte	Concentration (µg/mL)	Intra-day (n = 5)		Inter-day (n = 15)	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Gln	0.4	92.83	15.50	97.22	15.61
	1	102.7	9.080	97.45	9.500
	40	101.3	8.910	99.04	9.130
	200	106.7	7.810	102.8	8.990
Glu	0.2	92.03	9.420	96.28	13.21
	0.5	101.2	9.320	100.4	8.220
	5	98.92	3.910	98.16	8.790
	20	95.74	2.360	97.21	8.380
Ala	0.5	104.6	12.44	102.2	11.18
	1	102.7	11.13	98.85	8.910
	50	102.5	6.760	104.5	6.980
	100	95.33	12.29	97.16	10.76
Asp	0.2	113.4	9.740	106.5	12.10
	0.4	104.7	7.200	101.6	8.790
	10	101.0	8.220	100.8	6.970
	40	89.47	4.570	98.67	10.55
Akg	1	104.7	15.87	99.15	15.30
	2	97.92	11.04	99.50	8.140
	20	108.0	10.92	103.2	10.62
	100	105.0	5.360	102.9	8.740
Suc	0.2	101.6	12.13	104.4	11.68
	0.5	96.45	11.99	97.02	9.020
	2	90.04	5.420	98.66	10.07
	10	93.71	6.020	98.85	8.830
Fum	0.05	101.6	16.53	100.3	14.96
	0.1	100.5	8.360	99.88	9.470
	2	103.6	10.72	101.2	8.810
	10	94.58	9.430	100.2	8.750
Mal	0.5	98.93	16.63	100.6	13.57
	1	100.5	9.880	100.8	9.030
	10	103.4	9.760	95.75	9.660
	100	108.6	5.690	104.9	8.700
Cis-act	0.5	98.16	18.38	100.8	15.00
	1	98.34	7.050	96.64	5.920
	50	107.3	7.670	100.5	8.880
	200	98.13	10.79	96.85	7.070
Pyr	0.5	100.9	11.70	102.7	7.990
	1	100.4	4.190	101.6	5.820
	50	108.5	10.45	101.1	10.90
	200	97.64	8.160	98.00	9.780
Lac	8	95.73	12.69	97.22	13.18
	20	97.48	12.96	101.2	9.450
	200	97.47	12.06	101.2	9.580
	800	89.24	8.310	90.54	6.520
Cit	10	95.14	14.24	98.26	11.93
	20	103.2	8.590	100.5	8.460
	100	102.3	6.040	97.80	7.050

Table 4 (continued)

Analyte	Concentration (μg/mL)	Intra-day ($n = 5$)		Inter-day ($n = 15$)	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
	400	103.7	8.330	103.3	6.760
Iso-cit	0.5	95.22	12.64	103.7	10.95
	1	96.36	9.180	100.6	9.890
	5	102.8	11.04	105.4	7.130
	20	91.19	6.060	101.8	9.000

Shimadzu Nexera UFLC system coupled to an MS-8040 triple quadrupole mass spectrometer (Shimadzu Co., Tokyo, Japan) equipped with ESI source was applied to assess robustness. Three critical parameters including flow rate (± 0.2 mL/min), mobile phase additive concentration (± 0.2 mM), and column temperature (± 2 °C) were selected to be examined using MQC samples. The design of robustness experiment based on the settings is given in Table 2. The nominal value is the retention time (RT) in condition1 and RT variation (%) was calculated by the ratio of RT in different conditions to the nominal value. In addition, another new ZIC-*p*HILIC column was investigated to evaluate method robustness.

Participants and sample collection

T2D patients ($n = 34$) and healthy volunteers ($n = 38$) were recruited at the Second Affiliated Hospital of Harbin Medical University (Harbin, China) between March 2017 and June 2017. The detailed clinical information is shown in Table 3. The morning fasting antecubital venous blood plasma samples were collected and stored at − 80 °C until use. All participants voluntarily gave written informed consent and joined this study. The study protocol was approved by the Medical Ethics Committee of the Second Affiliated Hospital of Harbin
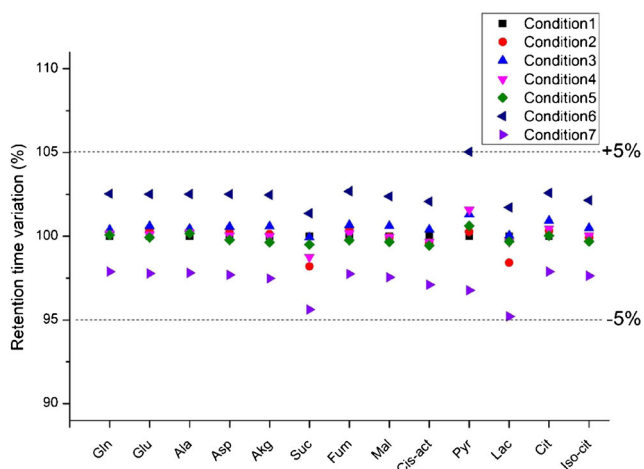
Medical University and was conducted according to the Helsinki Declaration of 1964, as revised in 1975.

Statistical analysis

All statistical analyses were performed using SPSS statistics 22.0 software (IBM SPSS Inc., USA). Differences in clinical and laboratory continuous variables were compared using Student's *t* test or Mann-Whitney test, depending on data normality. Categorical variables were compared using χ^2 test. $p < 0.05$ was considered significant.

Results and discussion

Optimization of chromatographic conditions

In this study, we chose 13 metabolites included since they are degradation products of glutaminolysis. Targeted metabolites were compounds with highly polar and weakly retained on a reversed-phase column. Therefore, the optimization of LC condition was primarily focused on evaluating the performance of different columns with stationary phases spanning a reversed-phase (RP) column and two hydrophilic interaction liquid chromatography (HILIC) columns. It was found that the Agilent ZORBAX SB-Aq column failed to provide effective retention for most targeted analytes, not to mention separating them well. The Waters XBridge® BEH Amide column as an alternative mode provided good separations for organic acids (except Cit and Iso-cit) but poor peak shapes for amino acids. Utilization of HILIC has grown in popularity for retaining and separating polar compounds [23]. The preliminary retention and separation of all 13 analytes were obtained on a Merck ZIC®-*p*HILIC column that carried densely bonded, zwitterionic functional groups with a charge balance of 1:1. Notably, the separation (resolution, $R = 1.952$) of isomers, Cit and Iso-cit, was also achieved on this column, probably due to their different solubility in acetonitrile (see Electronic Supplementary Material (ESM) Fig. S1).

To elute all analytes with high efficiency, combinations of different kinds of mobile phase additives, ionic strength, pH,

Fig. 2 Retention time variation of targeted metabolites in each condition

Fig. 3 The typical extracted-ion chromatogram (EIC) of each analyte obtained under optimal LC-ESI-MS/MS conditions in human plasma

Optimization of tandem mass spectrometry conditions
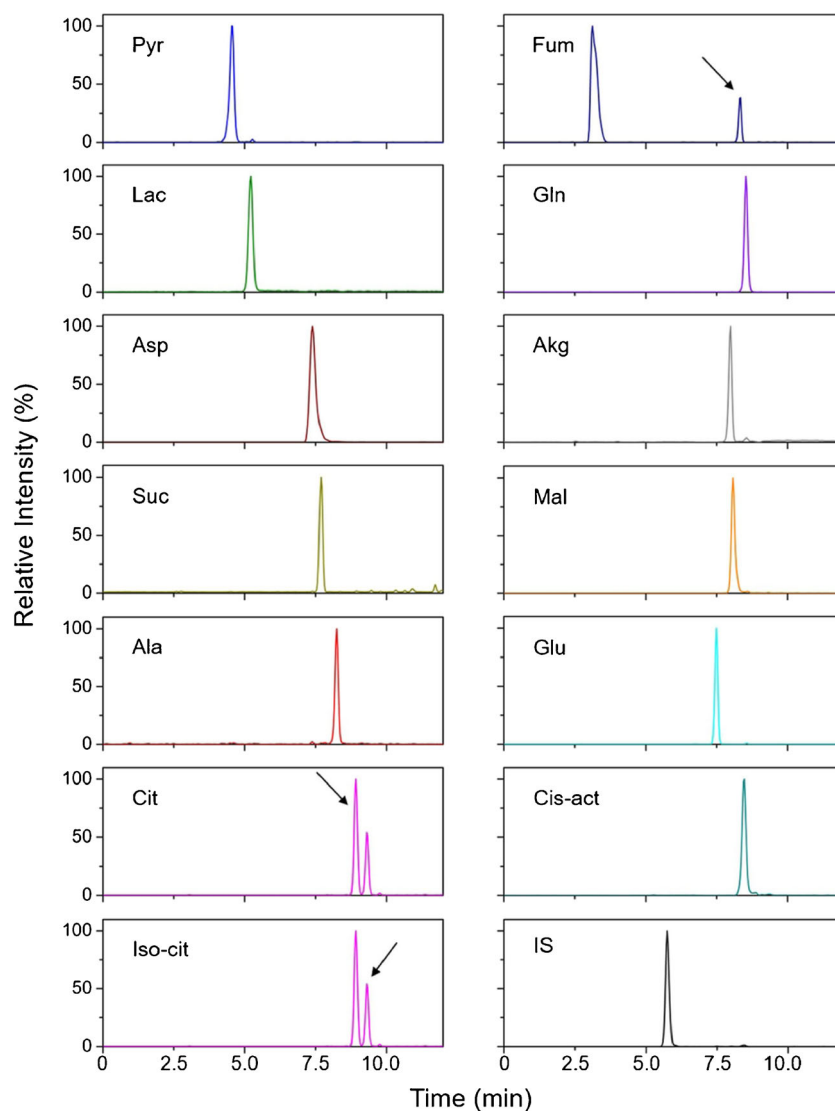
and gradient were investigated. Based on the key chromatographic parameters, such as resolution, symmetry factor, and peak width, five aqueous solvents used as solvent B were evaluated, including 0.1% formic acid; 0.1% ammonium hydroxide; 5 mM, 10 mM, and 15 mM ammonium acetate; 5 mM ammonium formate; and 5 mM ammonium bicarbonate in water. Among these additives, 5 mM ammonium acetate gave all analytes the best chromatographic behavior. To examine the influence of pH, tests were conducted on a series of pH values from 3.5 to 9.5. It was found that the MS responses of most targeted metabolites enhanced as the alkalinity of the solvent B increased (see ESM Fig. S2), whereas the isomers Cit and Iso-cit could not be chromatographically separated when pH ≥ 7.5 (ESM Fig. S3). To keep a balance between the sensitivity and specificity, the pH value at 6.8 in solvent B was selected. Eventually, the flow gradient was optimized to achieve the elution of 13 metabolites and IS in 10 min and the total analysis time of a single run of 20 min.

MS/MS operation parameters were carefully optimized to obtained high selectivity and sensitivity. It was observed that four amino acids (Ala, Asp, Glu, and Gln) could be ionized both in positive and negative ESI modes, while other analytes could only be detected in negative mode due to their acidic nature. Therefore, to avoid switching between the two modes in a single run, the negative mode was employed for all metabolites as well as IS. By flow injecting a neat standard solution which contains 1 μg/mL of the individual analyte, $[M-H]^-$ was found the richest relative abundance as the precursor ion in the full-scan spectra. Then, the product ion of each analyte was obtained by optimizing collision energies, spray voltage, capillary temperature, sheath gas pressure, and auxiliary gas pressure.

Table 5 Results of LC-MS/MS analysis of plasma samples from healthy volunteers and patients with T2D

Analyte	Plasma concentration (mean ± SD, μg/mL)		p value[a]
	T2D ($n = 34$)	Control ($n = 38$)	
Gln	75.14 ± 17.96	63.90 ± 20.73	0.0162
Glu	7.770 ± 2.720	5.100 ± 1.728	0.0000
Ala	24.74 ± 5.732	23.89 ± 4.947	0.4975
Asp	10.11 ± 2.630	9.937 ± 3.408	0.8149
Akg	9.070 ± 3.302	8.572 ± 2.629	0.4792
Suc	0.5009 ± 0.2643	0.6830 ± 0.3880	0.0219
Fum	0.2231 ± 0.1691	0.3868 ± 0.3078	0.0063
Mal	6.882 ± 2.481	8.279 ± 4.134	0.0836
Cis-act	3.692 ± 0.9345	2.800 ± 1.160	0.0006
Pyr	15.69 ± 3.835	16.69 ± 4.031	0.2851
Lac	345.7 ± 59.39	299.7 ± 58.15	0.0014
Cit	28.78 ± 8.045	31.66 ± 12.16	0.2453
Iso-cit	0.9206 ± 0.2473	0.7365 ± 0.2611	0.0031

[a] $p < 0.05$ was considered statistically significant

Noticeably, when monitoring the fragment ion at m/z 111.0 which was used by reported work [24], the difference in MS response between Cit and Iso-cit in human plasma was so great (~ 30 times) that the peak of Iso-cit was almost merged by the peak of Cit. After the optimization, we found a new fragment ion at m/z 172.9 providing similar MS responses to two isomers in the real sample (Fig. 1).

Method validation

The calibration curve of each analyte within the linear range exhibited good linearity (correlation coefficient, $r \geq 0.9911$). The LLOQ, representative regression equations of the calibration curves, and the correlation coefficients relating to the 13 metabolites are detailed in Table 1. The intra-day and inter-day precisions and accuracies at four concentration levels are summarized in Table 4. For low, middle, and high QC levels, the accuracy was between 89.24 and 108.6% and the precision of each analyte was less than 12.96%. With regard to the LLOQ, the precisions and accuracies for all metabolites were within 18.38% and between 92.03 and 113.4%, respectively. The robustness result (Fig. 2) of RT variation was between 95.2 and 105.0% in each condition, and the resolution of the isomer was at least 1.615 (see ESM Fig. S5), which confirmed the robustness of the method. Moreover, the new column exhibited similar chromatographic retention behaviors to the former. These results all indicated that the method was reliable, accurate, and reproducible.

By sequentially injecting the blank solution MeOH/ACN (50/50, v/v) after the ULOQ sample, no obvious MS response was observed in the chromatographic profiles at the RT of each analyte (see ESM Fig. S4), which indicated no carryover from residues in samples. The stability tests were designed to cover the anticipated conditions of handling of the real samples. As shown in ESM Table S1, the variations within 15.30% demonstrated that the analytes in plasma samples were stable when stored under the
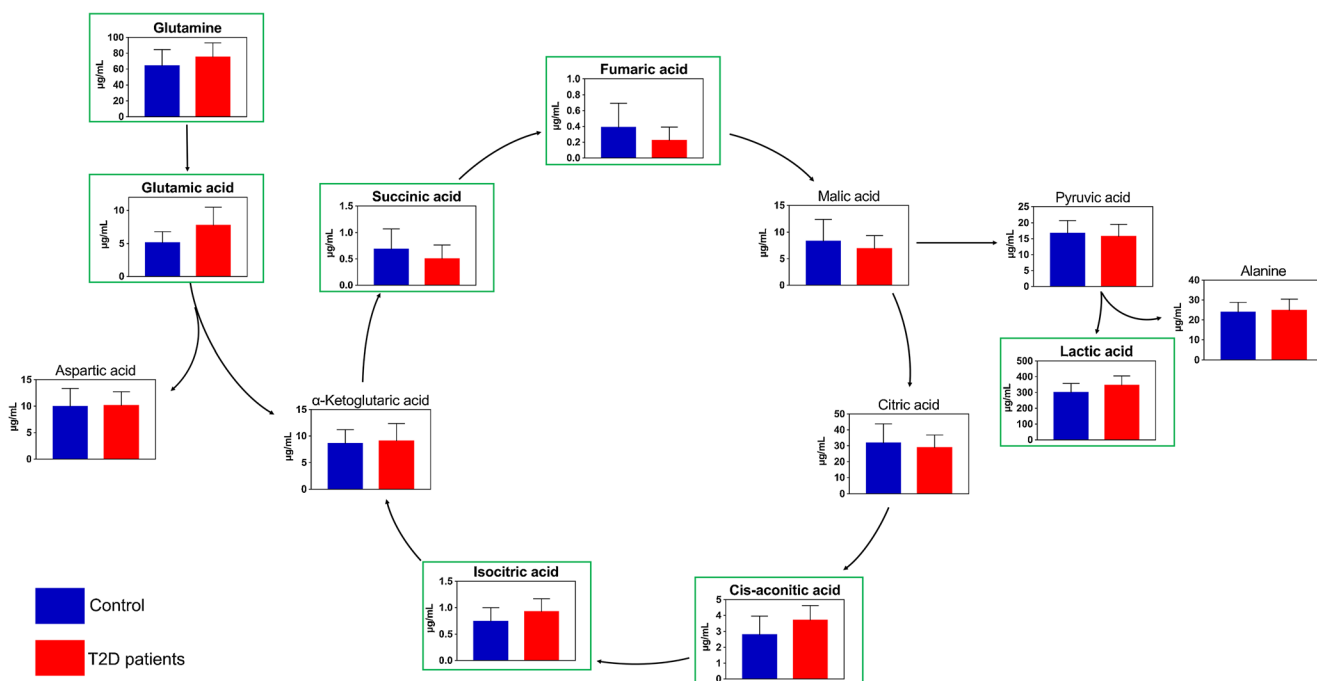

Fig. 4 A diagram for the significantly difference in glutaminolysis pathway of T2D patients compared with healthy controls. Metabolites with significant differences are represented by green boxes

conditions, including short-term, long-term, autosampler, and three freeze-thaw cycles. Any possible matrix effect was also studied and no significant suppression or enhancement was observed (see ESM Table S2). Recovery of QC samples ranged from 85.25 to 97.65% (see ESM Table S3), indicating the reproducibility and efficiency of sample preparation processes.

Method application

The validated method was applied to analyze the plasma samples of T2D patients and healthy controls. The typical extracted-ion chromatogram (EIC) of each analyte in human plasma is shown in Fig. 3. The quantitative results are summarized in Table 5. Student's t test revealed that 7 of the 13 metabolites in glutaminolysis showed significant differences in plasma levels between T2D and control samples, of which p values were less than 0.05. Among the 7 differential metabolites, the concentrations of Gln and Glu were upregulated in plasma samples of T2D patients, confirming the results with the previous report [10]. Our novel finding is that the effect of T2D on glutamine metabolism is further extended to the whole TCA cycle, resulting in lower levels of Fum and Suc and higher levels of Cis-act and Iso-cit (Fig. 4).

In TCA cycle, aconitase is an essential enzyme catalyzing the stereo-specific isomerization of Cit to Iso-cit via Cis-act [25]. Prior efforts have found that aconitase might be a potential therapeutic target for diabetes by normalization of aconitase activity in the lung [26]. However, the published LC-MS-based methods could not completely separate Cit and Iso-cit [27, 28], causing the role of aconitase to be ignored. In this work, abnormalities in concentrations of not only 11 polar metabolites but also a pair of isomers were distinguished and observed by the proposed method, showing its great value in exploring biomarkers and metabolic mechanisms of diseases involving changes in glutaminolysis.

Conclusions

In this study, a novel LC-MS/MS method using a pHILIC column was developed and validated for the determination of 13 metabolites in glutaminolysis in human plasma. The key parameters of separation and detection were systematically investigated and optimized. Compared with previously reports, key advantages of this method included are easy sample pre-treatment (required only 1 precipitation step), fast chromatographic separation (10 min), and wide dynamic range (0.05–800 μg/mL). This method also successfully achieved the separation and quantification for the isomers of Cit and Iso-cit. Validation results confirmed the robustness, reliability, and accuracy of this method. Moreover, the developed method was successfully applied to study the alters of glutaminolysis in T2D patients and healthy controls, demonstrating its capability in biomarker discovery and mechanism exploration.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were following the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from the individual participants who provided the blood samples.

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References

1. Durán RV, Oppliger W, Robitaille AM, Heiserich L, Skendaj R, Gottlieb E, et al. Glutaminolysis activates Rag-mTORC1 signaling. Mol Cell. 2012;47(3):349–58.

2. Le A, Lane AN, Hamaker M, Bose S, Gouw A, Barbi J, et al. Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in b cells. Cell Metab. 2012;15(1):110–21.

3. Altman BJ, Stine ZE, Dang CV. From Krebs to clinic: glutamine metabolism to cancer therapy. Nat Rev Cancer. 2016;16:619–34.

4. Jin L, Alesi GN, Kang S. Glutaminolysis as a target for cancer therapy. Oncogene. 2015;35(28):3619–25.

5. Villar VH, Nguyen TL, Delcroix V, Terés S, Bouchecareilh M, Salin B, et al. mTORC1 inhibition in cancer cells protects from glutaminolysis-mediated apoptosis during nutrient limitation. Nat Commun. 2017;8:14124.

6. Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. Trends Biochem Sci. 2011;35(8):427–33.

7. Dang CV. Glutaminolysis: supplying carbon or nitrogen or both for cancer cells? Cell Cycle. 2010;9(19):3884–6.

8. Jacque N, Ronchetti AM, Larrue C, Meunier G, Birsen R, Willems L, et al. Targeting glutaminolysis has antileukemic activity in acute myeloid leukemia and synergizes with BCL-2 inhibition. Blood. 2015;126(11):1346–56.

9. Kelly A, Li C, Gao Z, Stanley CA, Matschinsky FM. Glutaminolysis and insulin secretion from bedside to bench and back. Diabetes. 2002;51((suppl3)(6)):S421–6.

10. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC, Ong CN. Metabolic signature shift in type 2 diabetes mellitus revealed by mass spectrometry-based metabolomics. J Clin Endocrinol Metab. 2013;98(6):1060–5.
11. Zhang AH, Sun H, Yan GL, Yuan Y, Han Y, Wang XJ. Metabolomics study of type 2 diabetes using ultra-performance LC-ESI/quadrupole-TOF high-definition MS coupled with pattern recognition methods. J Physiol Biochem. 2014;70(1):117–28.
12. Siegel D, Permentier H, Reijngoud DJ, Bischoff R. Chemical and technical challenges in the analysis of central carbon metabolites by liquid-chromatography mass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci. 2014;966:21–33.
13. Jung JY, Oh MK. Isotope labeling pattern study of central carbon metabolites using GC/MS. J Chromatogr B Anal Technol Biomed Life Sci. 2015;974:101–8.
14. Nemkov T, Hansen KC, D'Alessandro A. A three-minute method for high-throughput quantitative metabolomics and quantitative tracing experiments of central carbon and nitrogen pathways. Rapid Commun Mass Spectrom. 2017;31(8):663–73.
15. Hur H, Paik MJ, Xuan Y, Nguyen DT, Ham IH, Yun J, et al. Quantitative measurement of organic acids in tissues from gastric cancer patients indicates increased glucose metabolism in gastric cancer. PLoS One. 2014;9(6):1–9.
16. Calderón-Santiago M, Priego-Capote F, Galache-Osuna JG, Luque de Castro MD. Method based on GC-MS to study the influence of tricarboxylic acid cycle metabolites on cardiovascular risk factors. J Pharm Biomed Anal. 2013;74:178–85.
17. Hnatyshyn S, Shipkova P. Automated and unbiased analysis of LC-MS metabolomic data. Bioanalysis. 2012;4(5):541–54.
18. Al Kadhi O, Melchini A, Mithen R, Saha S. Development of a LC-MS/MS method for the simultaneous detection of tricarboxylic acid cycle intermediates in a range of biological matrices. J Anal Methods Chem. 2017;2017(5391832):1–12.
19. Michopoulos F, Whalley N, Theodoridis G, Wilson ID, Dunkley TPJ, Critchlow SE. Targeted profiling of polar intracellular metabolites using ion-pair-high performance liquid chromatography and -ultra high performance liquid chromatography coupled to tandem mass spectrometry: applications to serum, urine and tissue extracts. J Chromatogr A. 2014;1349:60–8.
20. Luo B, Groenke K, Takors R, Wandrey C, Oldiges M. Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle by liquid chromatography-mass spectrometry. J Chromatogr A. 2007;1147:153–64.
21. Han J, Gagnon S, Eckle T, Borchers CH. Metabolomic analysis of key central carbon metabolism carboxylic acids as their 3-nitrophenylhydrazones by UPLC/ESI-MS. Electrophoresis. 2013;34(19):2891–900.
22. González O, Blanco ME, Iriarte G, Bartolomé L, Maguregui MI, Alonso RM. Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect. J Chromatogr A. 2014;1353:10–27.
23. Buszewski B, Noga S. Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique. Anal Bioanal Chem. 2012;402(1):231–47.
24. Fernández-Fernández R, López-Martínez JC, Romero-González R, Martínez-Vidal JL, Alarcón Flores MI, Garrido Frenich A. Simple LC–MS determination of citric and malic acids in fruits and vegetables. Chromatographia. 2010;72(1–2):55–62.
25. Wang P, Mai C, Wei Y, Zhao J, Hu Y, Zeng Z, et al. Decreased expression of the mitochondrial metabolic enzyme aconitase (ACO2) is associated with poor prognosis in gastric cancer. Med Oncol. 2013;30(552):1–9.
26. Gumieniczek A, Komsta Ł, Chehab MR. Effects of two oral anti-diabetics, pioglitazone and repaglinide, on aconitase inactivation, inflammation and oxidative/nitrosative stress in tissues under alloxan-induced hyperglycemia. Eur J Pharmacol. 2011;659(1):89–93.
27. Bajad SU, Lu W, Kimball EH, Yuan J, Peterson C, Rabinowitz JD. Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography-tandem mass spectrometry. J Chromatogr A. 2006;1125(1):76–88.
28. Fiori J, Amadesi E, Fanelli F, Tropeano CV, Rugolo M, Gotti R. Cellular and mitochondrial determination of low molecular mass organic acids by LC-MS/MS. J Pharm Biomed Anal. 2017;150:33–8.