

ORIGINAL ARTICLE

# Analysis of tricarboxylic acid cycle intermediates in dried blood spots by ultra-performance liquid chromatography-tandem mass spectrometry

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## ABSTRACT

**Background:** We developed a novel method for measuring the concentrations of tricarboxylic acid (TCA) cycle intermediates in dried blood spots (DBS) using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Analytes were derivatized before analysis using 4-[2-(N,N-dimethylamino) ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxa-diazole (DAABD-AE), a reagent that imparts powerful chromatographic and mass spectrometric properties onto carboxyl group-containing analytes.

**Methodology:** Extraction and derivatization of TCA cycle intermediates were achieved in a single step by incubating a 3.2 mm circle of the DBS samples with DAABD-AE for 1 hour at 65°C. From the resultant mixture, 1.0 µl was injected into the LC-MS/MS.

**Results:** Total analytical run time to separate target analytes from other interfering components in the sample was 8 minutes. The peaks corresponding to malic, fumaric, citric, succinic, and 2-ketoglutaric acid appeared at 3.55, 3.62, 3.64, 3.67, and 3.68 minutes, respectively. The method was adequately reproducible with a coefficient of variation for intraday ( $n = 15$ ) and inter-day ( $n = 13$ ) studies of 5.2%–18.4%. Reference intervals in DBS from controls ( $n = 125$ ) were as follow (µmol/l): citric (36.6–126.4), 2-ketoglutaric (9.1–42.1), succinic (1.2–2.4), fumaric (2.4–9.0), and malic acid (15.9–39.3). Compared to controls, the levels of citric, succinic, and malic acids were statistically different in patients ( $n = 7$ ) with a  $p$ -value of  $< 0.05$ . No statistically significant difference was detected in concentrations of 2-ketoglutaric and fumaric acids.

**Conclusion:** We describe a simple, quick, and sensitive method to measure TCA cycle intermediates in DBS samples. That TCA cycle plays a central role in cellular metabolism; this method should be useful in studying these metabolites in health and disease.

**Keywords:** Tricarboxylic acid cycle, citric acid, 2-ketoglutaric acid, succinic acid, fumaric acid, malic acid, DBS, LC-MS/MS, DAABD-AE.

## Introduction

The tricarboxylic acid (TCA) cycle, which takes place in the mitochondria, is the common oxidative pathway for fuel molecules. This cycle is ubiquitously expressed in body organs, including renal, hepatic, and cardiovascular and central nervous systems. Involving a series of oxidation-reduction reactions, the TCA cycle contributes to energy generation using aerobic respiration to fulfill the energy required for metabolism, growth, development, and other physiological processes. Metabolic intermediates of lipids, carbohydrates, and amino acids enter the TCA cycle and are further metabolized in a series of enzymatic conversions to produce energy. Therefore, evaluating the

disruptions of TCA cycle intermediates may provide clues to understand the basis of a multitude of disorders that affect mitochondrial energy production (1,2).

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**Received:** 17 July 2019 | **Accepted:** 12 December 2019

Metabolic disorders involving the TCA cycle, including deficiencies of  $\alpha$ -ketoglutarate dehydrogenase, succinate dehydrogenase, and fumarase constitute a rare but important subgroup of human diseases with a wide range of severity and clinical manifestations. These include organ systems with high energy demand that are mainly affected by these disorders (3). Mutations in specific TCA cycle genes such as those encoding fumarate hydratase and succinate dehydrogenase have been linked to certain types of tumors (4,5). Experimental studies on animals have found links between Alzheimer's disease and metabolic disruptions in the TCA cycle in astrocytes (6). Therefore, developing sensitive and specific methods to study TCA cycle metabolites in biological samples could be a useful approach for exploring the consequences of genetic and environmental influences on this pivotal metabolic pathway (7). Gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are the major techniques employed for the analysis of TCA cycle intermediates in biological samples (2,8,9). Combining the power of chromatographic resolution and mass spectrometry, these methods provide qualitative and quantitative information on the studied analytes. Therefore, they are preferred over other methodologies such as enzymatic and nuclear magnetic resonance-based methodologies (10). However, to date, there is no published protocol on the quantification of TCA cycle intermediates in dried blood spot (DBS) samples. DBS is a superior sample type compared with urine or plasma due to the sample collection, transport, and storage requirement. Furthermore, biomarkers on DBS are sufficiently stable making this minimally invasive sample the matrix of choice for various analyses (11,12). In this paper, we developed and validated a novel method to measure specific TCA cycle intermediates in DBS using LC-MS/MS. Citric acid (CA), 2-ketoglutaric acid (KG), succinic acid (SA), fumaric acid (FA), and malic acid (MA) were derivatized with 4-[2-(N,N-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxa-diazole (DAABD-AE) (13). This derivatization in combination with LC-MS/MS allows the detection of very minute quantities of metabolites that are present in small concentrations (14–16). The developed method was adequately sensitive and allowed for multiplexed analysis of TCA cycle intermediates in DBS from healthy and diseased individuals.

## Subjects and Methods

### Chemicals and standard solutions

CA, KG, SA, DAABD-AE, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), perfluorooctanoic acid (PFOA), formic acid, hydrochloric acid, and 4-(dimethyl-amino) pyridine (DMAP) were obtained from Sigma Aldrich (Taufkirchen, Germany). Deuterium labeled CA (d4-CA) used as internal standard (IS) was purchased from Cambridge Isotopes Laboratories (Tewksbury, Massachusetts). MA and FA were procured

from Tokyo Chemical Industry (Toshima, Kita-Ku, Tokyo, Japan). LC-MS/MS grade acetonitrile, methanol and water were purchased from Merck (Darmstadt, Germany). Stock solutions of individual analytes and IS were prepared by dissolving appropriate amounts of each compound in 50% acetonitrile to attain a concentration of 1.0 mg/ml. Working solutions were prepared by dilution in the same solvent. The working d4-CA IS solution was prepared at 24  $\mu$ mol/l. All solutions were placed in tightly capped amber glass vials at 4°C and were stable for at least 4 months. A premixed solution containing the reagents required for derivatization (1:1:2 v/v/v) hereafter referred to as a master mix was prepared using freshly prepared EDC (25 mmol/l in water), DMAP (25 mmol/l in acetonitrile), and DAABD-AE (2 mmol/l in 90% Acetonitrile). The master mix was stable for at least 1 week when stored at 4°C.

### Control and patient samples

This study was approved by Al Ain Medical District Human Research Ethics Committee. The control DBS samples ( $n = 125$ ) used to generate the reference intervals in this study were obtained from healthy volunteers. A total of seven DBS samples from patients with propionic acidemia ( $n = 2$ ) and methylmalonic acidemia ( $n = 5$ ) were also analyzed. All samples were collected on a Whatman 903™ Specimen Collection Paper and allowed to dry at room temperature for 4 hours. Except during use, DBS samples were stored in sealed plastic bags at 4°C.

### Sample preparation

From a DBS, a 3.2-mm disc was excised and placed in a 1.5 ml Eppendorf Snap-Cap microcentrifuge tube. Aliquot of 10  $\mu$ l working IS solution was added to the Eppendorf tube, along with 100  $\mu$ l of the master mix. The tubes were capped and incubated in a digital dry bath for one hour at 65°C. The reaction was stopped using 400  $\mu$ l of mobile phase A. The tubes were centrifuged for 30 seconds at 13,000 rpm and 300  $\mu$ l of the supernatant were transferred to the designated well in a 96 well plate. Portions of 1.0  $\mu$ l were analyzed by LC-MS/MS.

### LC-MS/MS system

The samples were analyzed using a Nexera-i series LC-2040 liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of a quaternary solvent gradient pump, degasser, autosampler, column oven, and system controller. A Kinetex 1.7  $\mu$ m C8 100A LC column (50  $\times$  2.1 mm, Phenomenex) was used for the separation of analytes. Mobile phase A was prepared using 10% methanol and mobile phase B was 90% methanol both containing 0.05% PFOA. The column oven temperature was set at 40°C. Using a flow rate of 0.4 ml/minute, the following gradient was applied: 0–1 minute 2% B, 1–4 minutes 2%–85% B, and 4–6 minutes 85%–95% B. The column were re-equilibrated for 2 minutes with 2% B resulting in an injection-to-injection time of 8

minutes. The electrospray ionization source of the mass spectrometer (Model LC-MS 8060 triple quadrupole mass spectrometer, Shimadzu) was operated in positive ion mode with a capillary voltage of 4.5 kV. Argon was used for collision-induced dissociation. The desolvation and ion source temperatures were set at 250°C and 400°C, respectively. Detection of CA, KG, FA, MA, and SA was in the multiple reaction monitoring modes using the  $m/z$  transitions shown in Table 1.

### Method validation

DBS calibrators were prepared by spiking whole blood from healthy volunteers with standard analyte solutions. For CA and KG, the following concentrations were prepared: 5, 10, 25, 50, 100, 150, and 200  $\mu\text{mol/l}$ . For MA, the following concentrations were prepared: 2.5, 5, 12.5, 25, 50, 75, and 100  $\mu\text{mol/l}$ . For FA and SA, the following concentrations were prepared: 0.25, 0.5, 1.25, 2.5, 5, 7.5, and 10  $\mu\text{mol/l}$ . To account for endogenous levels, blood without the addition of standard solutions was included in each batch. Quality control (QC) samples at low, medium, and high concentrations were prepared using whole blood from healthy volunteers. Calibrators and QCs were applied onto the Whatman 903<sup>TM</sup> Specimen Collection Paper and allowed to dry at room temperature for at least 4 hours. Except during use, DBS specimens were stored in sealed plastic bags at 4°C. Intraday ( $n = 15$ ) and interday ( $n = 13$ ) variations were assessed by analyzing the low, medium, and high QC samples. The

coefficients of variation (CV%) of these measurements were calculated according to the equation  $[\text{CV}\% = 100 \times \text{standard deviation}/\text{mean}]$ .

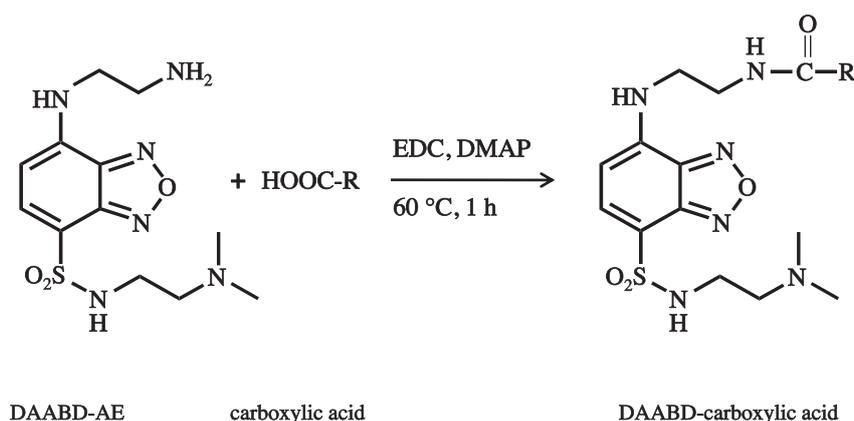
## Results and Discussion

### Sample preparation

Organic acids including TCA cycle intermediates are small, polar metabolites with poor analytical properties. In particular, these analytes have poor chromatographic and mass spectrometric characteristics. To overcome this, derivatization with DAABD-AE was undertaken in this work. Not only had this resulted in derivatives that are compatible with reversed-phase chromatography, but it also imparted suitable features for positive ion electrospray ionization MS/MS detection by introducing a site with high proton affinity (14–16). The derivatization reaction scheme of organic acids with DAABD-AE is shown in Figure 1. The optimum derivatization reaction conditions with DAABD-AE were determined using standard solutions of TCA cycle intermediates. Reaction time intervals of 10, 20, 30, 40, 50, 60, 70, 80, and 90 minutes were examined and 60 minutes was found to be the optimal time at which the derivatization reaction yield reached a plateau. Among the various reaction temperatures evaluated (i.e., 23°C, 65°C, and 90°C), the optimal temperature with maximum reaction yield was 65°C. In subsequent experiments, the derivatization of TCA cycle intermediates was performed by incubation of

**Table 1.** LC-MS/MS parameters of TCA cycle intermediates.

Analyte	Precursor ion ( $m/z$ )	Production ion ( $m/z$ )	Collision energy (eV)	Dwell time (sec)	Retention time (min)
CA	485.1	151.10	-25	0.097	3.64
KG	457.1	151.10	-25	0.097	3.68
SA	429.1	151.10	-25	0.097	3.67
FA	427.0	151.10	-25	0.097	3.62
MA	445.0	151.10	-25	0.097	3.55
d4-CA	489.1	151.10	-25	0.097	3.64



**Figure 1.** The scheme of derivatization reaction of carboxylic acids including TCA cycle intermediates with DAABD-AE.

3.2 mm DBS disc with the IS and 100  $\mu$ l of the master mix at 65°C for 1 hour. The superior reactivity of DAABD-AE allowed for a single step sample preparation (i.e., extraction from DBS and formation of DAABD-TCA cycle intermediates derivatives). This is in agreement with our previously reported results (16). The derivatives were found to be stable for approximately 72 hours when stored at 4°C away from direct light.

### LC-MS/MS analysis

Figure 2 shows the representative chromatographic separation of TCA cycle intermediates achieved by this method. Using a reversed-phase C8 column, the studied analytes were adequately retained and separated from early and late eluting substances that may have ion suppression effects. The use of d4-CA facilitated the assignment of the CA peak. Under the conditions and  $m/z$  transitions employed in this work, a single well-resolved peak was detected for each of SA and MA. On the other hand, two peaks were detected at the  $m/z$  of KG and FA, however, the correct peak was ascertained by evaluating the detector response using variable analyte concentrations. All target analytes eluted from the column in less than 4.0 minutes in the following order: MA, FA, CA, SA, and finally KG (Figure 2). In this work, column flushing and re-equilibration steps were included; therefore, the injection-to-injection time was 8 minutes. This relatively short analysis time allows for the application of this method in a high throughput setting.

### Method validation

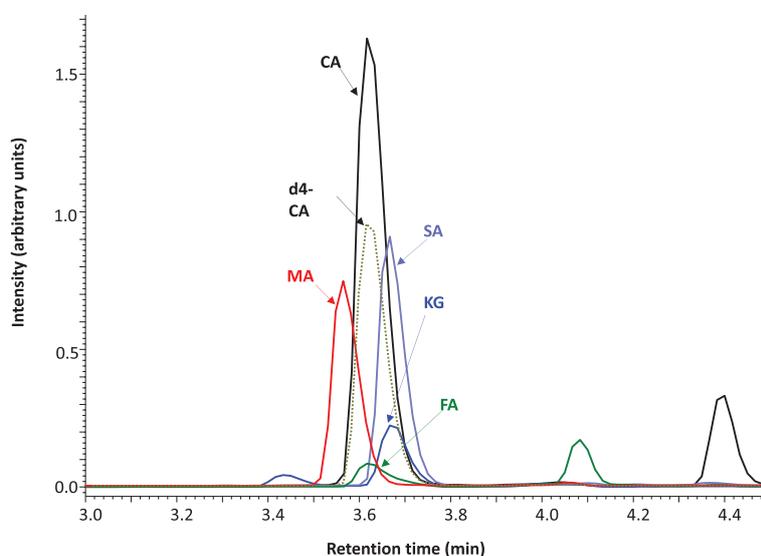
Linearity was evaluated using whole blood samples spiked with TCA cycle intermediates and used to create DBS calibrators. Because endogenous levels of these analytes are widely variable, the concentration ranges

to be investigated were selected to mimic those usually found in physiological and pathological conditions. In this study, the linearity of CA (endogenous + spiked) was confirmed up to 296  $\mu$ mol/l, KG (endogenous + spiked) was confirmed up to 206.7  $\mu$ mol/l, SA (endogenous + spiked) was confirmed up to 11.5  $\mu$ mol/l, FA (endogenous + spiked) was confirmed up to 13.7  $\mu$ mol/l and MA (endogenous + spiked) was confirmed up to 118.4  $\mu$ mol/l. The correlation coefficient ( $r$ ) of the curves obtained for all compounds ranged between 0.989 and 0.999. Intraday and interday reproducibility were evaluated at three different concentrations for each analyte. The CV (%) of intraday ( $n = 15$ ) and interday ( $n = 13$ ) imprecision studies evaluated obtained in this study ranged from 5.2% to 18.4% as shown in Table 2.

### TCA cycle intermediates in control and patients samples

Table 3 shows the reference intervals of the TCA cycle intermediates that were determined in this work using DBS from healthy control subjects ( $n = 125$ ). CA, KG, and MA are present at concentrations relatively higher than those of SA and FA in control DBS samples.

In this work, we analyzed samples from patients ( $n = 7$ ) with inborn errors of metabolism known to affect the TCA cycle including propionic acidemia ( $n = 2$ ) and methylmalonic acidemia ( $n = 5$ ). In these patients, the CA range was noted to be lower compared to controls. This is anticipated because, in these patients, the pathologically accumulating propionyl-CoA competes with acetyl-CoA and consumes oxaloacetate in the TCA cycle and results in lower CA levels. Other analytes namely KG, SA, and FA were higher in patients compared to controls. A two-tailed t-test revealed a statistically significant difference between the two groups ( $p$ -value < 0.05) for CA, SA,



**Figure 2.** Extracted mass chromatograms obtained with a DBS from a healthy individual.

**Table 2.** Intraday and interday reproducibility of TCA cycle intermediates in DBS.

Sample	Compound	Concentration added ( $\mu\text{mol/L}$ )	Intraday ( $n = 15$ )		Interday ( $n = 13$ )	
			Mean ( $\mu\text{mol/l}$ )	CV (%)	Mean ( $\mu\text{mol/l}$ )	CV (%)
QC1	CA	0	96.0	7.5	98.3	10.8
	KG	0	9.9	8.9	7.4	5.2
	SA	0	3.5	10.0	3.5	12.3
	FA	0	0.8	9.2	1.3	18.4
	MA	0	12.9	8.8	15.5	14.8
QC2	CA	100	193.9	10.3	201.4	12.6
	KG	5	12.8	13.5	13.4	15.2
	SA	5	6.0	11.6	6.3	13.8
	FA	5	8.6	15.7	10.5	15.9
	MA	50	61.5	11.7	77.6	12.7
QC3	CA	150	247.8	8.7	274.7	11.6
	KG	10	16.2	12	18.0	14.8
	SA	10	10.0	9.7	11.0	13.3
	FA	10	10.3	7.9	14.6	12.0
	MA	100	93.4	10.1	114.0	14.0

**Table 3.** Concentrations of TCA cycle intermediates in DBS samples from patients and controls

		Concentration ( $\mu\text{mol/l}$ )				
		CA	KG	SA	FA	MA
Patients ( $n = 7$ )	Median	56.5	15.4	2.6	6.4	21.1
	Range	34.3–72.0	10.9–57.0	2.0–5.0	41–12.5	17.2–30.2
Controls ( $n = 125$ )	Median	73.4	16.1	1.6	5.7	28.2
	Reference interval*	36.6–126.4	9.1–42.1	1.2–2.4	2.4–9.0	15.9–39.3
$p$ -value		0.0219**	0.2000	9.069E-14**	0.1307	0.0090**

\*25%–97.5%.

\*\* Significance.

and MA. On the other hand,  $p$ -values of more than 0.05 were obtained for KG and FA indicating no statistically significant difference in the concentration of these analytes between the two groups. This might have been caused by the limitation of using a small number of patients and the nature of inborn errors of metabolism studied in this work.

## Conclusion

This article describes the development and validation of a new method to determine the TCA cycle intermediates CA, KG, SA, FA, and MA in DBS. To our knowledge, this is the first quantitative method of these analytes in DBS by LC-MS/MS. The method is based on DAABD-AE derivatization which significantly improved the chromatographic and mass spectrometric properties of the target analytes and allowed for multiplexed measurements in a small amount of blood contained in

a single 3.2 mm DBS disc. Sample preparation including extraction and derivatization was simple and completed in one step at 65°C for 60 minutes. The current method utilizes DBS samples that are superior to other sample types due to ease of collection, transport, and storage. Given the central role played by the TCA cycle in cellular metabolism, this method should be useful to study these metabolites in various health and disease settings.

## Acknowledgments

The authors are grateful to patients and their families for their contributions to this work.

## List of Abbreviations

CA	citric acid
CV%	coefficients of variation (%)
DAABD-AE	4-[2-(N,N-dimethylamino) ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxa-diazole

DBS	dried blood spot
DMAP	4-(dimethyl-amino) pyridine
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
FA	fumaric acid
KG	2-ketoglutaric acid
LC-MS/MS	liquid chromatography tandem mass spectrometry
MA	malic acid
PFOA	perfluorooctanoic acid
QC	quality control
SA	succinic acid
TCA	tricarboxylic acid

### Funding

This work was supported by UAE University grants: Sure Plus and 31M314.

### Declaration of conflicting interests

The authors of this article have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

### Ethical approval

Al Ain Medical District Human Research Ethics Committee granted approval for this study (ERH-2017–5494 17-01).

### Consent for publication

Informed consent was obtained from the parents.

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