Application Note

Clinical Research

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Measurement of D- and L-2-Hydroxyglutarate Enantiomers and *Alpha* Ketoglutaric Acid by LC/MS/MS

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Abstract

Recent updates to the hallmarks of cancer, have included metabolism with deregulated cellular bio energetics. Activating mutations in the metabolic enzyme isocitrate dehydrogenase (IDH) leading to the abnormal accumulation of 2-hydroxyglutaric acid (2-HGA) has been described in hematologic malignancies and some solid tumors including gliomas. However, not all tumors with higher levels of 2-HGA have IDH mutations, and instead accumulate a different enantiomer. The phenotype resulting from 2-HGA accumulation is related to its binding to *alpha* ketoglutarate (αKG)-dependent enzymes and inhibiting their activity. For this reason, the level of αKG is important, and previous reports have indicated it is the ratio of 2-HGA to αKG that best correlated with tumor phenotype. Derivatization of 2-HGA with (+)-*o,o*-diacetyl-l-tartaric anhydride (DATAN) permitted separation of the two resulting diastereoisomers without a chiral stationary phase, as well as underivatized αKG on a C18 column. The analysis was performed using an Agilent 1290 Infinity II LC connected to an Agilent 6490 triple quadrupole LC/MS with an Agilent Jet Stream ion source. This method produced linear standard curves over the range 0.34–135.04 µm with R^2 values >0.9 and low matrix effects.

Introduction

IDH is frequently mutated in cancer. IDH mutations have been reported in a wide range of human tumors, including hematopoietic malignancies as acute myeloid leukemia (AML), myelodysplastic syndrome (MPD), and myeloproliferative neoplasms (MPN). It is also reported in solid tumors as low-grade gliomas, secondary glioblastomas, intrahepatic cholangiocarcinomas, chondrosarcomas, and other tumor types at lower frequency[1]. IDH normally catalyzes a TCA cycle reaction, the oxidative decarboxylation of isocitrate to α-KG, which is coupled with nicotinamide adenine dinucleotide phosphate (NADPH) production[2,3]. This reaction is essential for oxidative stress response and several metabolic processes. Mutant IDH acquires an abnormal enzymatic activity, allowing it to convert αKG into 2-HGA, a so-called oncometabolite that accumulates in IDH-mutated cells[4,5].

In addition to IDH mutation, other mechanisms of 2-HGA accumulation have been identified. For example, an IDH mutation-independent mechanism identified in kidney cancer and other tumor types results in the accumulation of the (L)2-HGA enantiomer of 2-HGA. There are also nonmalignant disorders such as L-2-Hydroxyglutaric aciduria involving mutations in the gene encoding for L-2-Hydroxyglutarate Dehydrogenase leading to a neurometabolics disorder from the accumulation of (L)2-HGA.

The primary effect of 2-HGA is to inhibit αKG-dependent enzymes including DNA demethylases, leading to hypermethylation of DNA in these cells. (L)2-HGA is a more potent inhibitor of demethylases compared to (D)2-HGA. Therefore, the separation of (L) and (D) 2-HGA has importance in many diseases (Figures 1–3).

Experimental

Sample preparation

D-2-hydroxyglutaric acid and L-2-hydroxyglutaric acid were prepared at a concentration of 10 mM for calibration standards (STDs). Separately, approximately 10 µg of D-α-hydroxyglutaric acid-$^{13}C_5$ disodium salt was weighed out for the internal standard (IS). To obtain 10 mM stock solutions, water was added to each weighing.

Working solutions were then prepared containing 100 µM (D)2-HGA and (L)2-HGA in water for STDs and QCs. These working solutions were further diluted in water (v/v) to 0.05, 0.125, 0.25, 0.5, 1, 2, 5, 10, and 20 µM for the nine STDs, and 0.15, 0.3, 1.6, 8, and 16 g/mL for the five QCs. Corresponding concentrations, expressed in nM, were 0.338, 0.844, 1.69, 3.38, 6.75, 13.50, 33.76, 67.52, and 135.04 for the STDs, and 1.02, 2.03, 10.8, 54.02, and 108.03 for the QCs. The IS working solution was prepared at 50 ng/mL (390.3 nM) in water. All in-house calibrators were prepared in DC MS Gold Serum (Golden West Biological, Inc).

To each of these samples, 2 mL of HPLC grade ethyl acetate:hexane (3:2) was added, then vortexed for two minutes. Then, the upper organic layer was transferred to another clean tube with 500 µL of NaOH at 0.1 M, and vortexed. The organic layer was then collected and dried under nitrogen at room temperature. The samples, calibrators, controls, and blanks were subsequently reconstituted in 100 mL of 70:30 % water:methanol.

The strong anion exchange and reversed-phase STRATA XL-A (200 mg–3 mL) SPE cartridges were selected to maximize analyte recovery and minimize matrix effects. Each cartridge was conditioned with 2 mL of methanol, then equilibrated with 2 mL of water. The pretreated samples were then loaded onto the cartridges. Each was washed with 1 mL of 25 mM ammonium acetate solution, then 1 mL of methanol. The cartridges were dried, and analytes eluted with 2 mL of methanol containing 5 % formic acid. The 2 mL eluate was evaporated to dryness in a Savant Speedvac system SPD121P (Thermo Scientific, San Jose, CA, USA) at 80 °C.

To separate the two enantiomers without a chiral stationary phase, a chiral derivation of the hydroxyl group on the asymmetric carbon was necessary. For this purpose, a 50 mg/mL solution of DATAN in methylene chloride and acetic acid (4:1, v/v) was used. One hundred microliters of this freshly prepared solution were added to the dry residue; the vial was capped hermetically and heated at 75 °C for 30 minutes. This derivatization procedure (Figure 1) produced diastereomers of (L)2-HGA and (D)2-HGA, allowing their separation by reversed-phase chromatography. After cooling to room temperature, the mixture was evaporated to dryness at 37 °C. The dry residue was dissolved in 200 µL of 2 mM aqueous ammonium formate, pH 3.1, and transferred to an autosampler vial and capped. Ten microliters were injected into the LC column using the full loop mode.

Instrumentation

- Agilent 1290 Infinity II LC
- Agilent 6495 triple quadruple LC/MS

Parameter	Value
Ion source	Agilent Jet Stream
Ion polarity	Negative
Gas temperature	260 °C
Gas flow	11 L/min
Nebulizer	30 psi
Sheath gas temperature	400 °C
Sheath gas flow	12 L/min
Capillary voltage	4,000 V
Nozzle voltage	2,000 V
Q1/Q2 resolution	0.7/0.7 unit
Dwell time	80 msec
Delta EMV	+500 V
Fragmentor	380 V
Cell accelerator voltage	3

Data analysis software:
Agilent MassHunter quantitative analysis

Results and discussion

The method was adequately tested, and demonstrated good precision and recovery values with a limit of quantitation (LOQ) of 0.20 µM in collected samples (Figures 3 and 4).

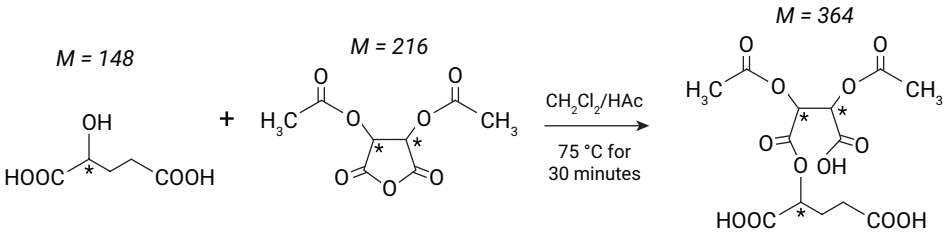

Figure 1. Derivatization of (D)2-HGA and (L)2-HGA with DATAN. Asymmetric carbon atoms are indicated with *.

Chromatographic conditions

Parameter	Value
Column	Agilent ZORBAX SB-C18 column (4.6 × 150 mm, 5 µm)
Column temperature	37 °C
Injection volume	10 µL
Needle wash	Flush port (50 % IPA, 20 % MeOH, 20 % ACN, 10 % water) 5 seconds
Mobile phase A	2 mM Ammonium formate aqueous solution, adjusted to pH 3.1 with formic acid
Mobile phase B	100 % Acetonitrile
Flow rate	0.3 mL/min

Gradient

Time (min)	%A	%B	Flow (mL/min)	Max. pressure limit (bar)
0	95	5	0.8	1,000
5.0	95	5	0.8	–
5.1	70	30	0.8	–
6.5	70	30	0.8	–
6.6	95	5	0.8	–
8.6	95	5	0.8	–

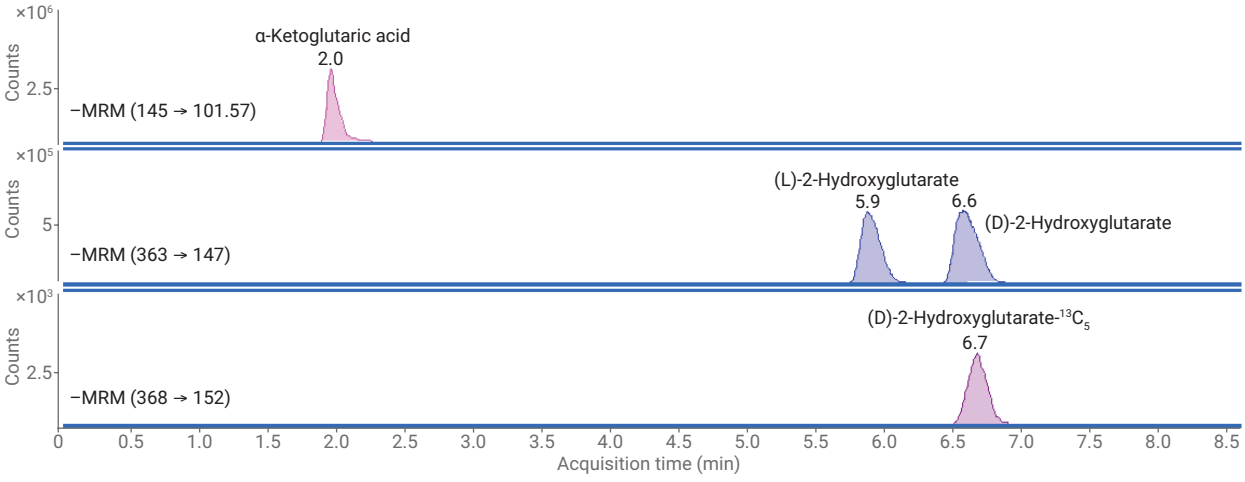

Figure 2. Standard MRM chromatograms representing the separation of (D) and (L)2-HGA, D-2-HGA-$^{13}C_5$, and αKG.

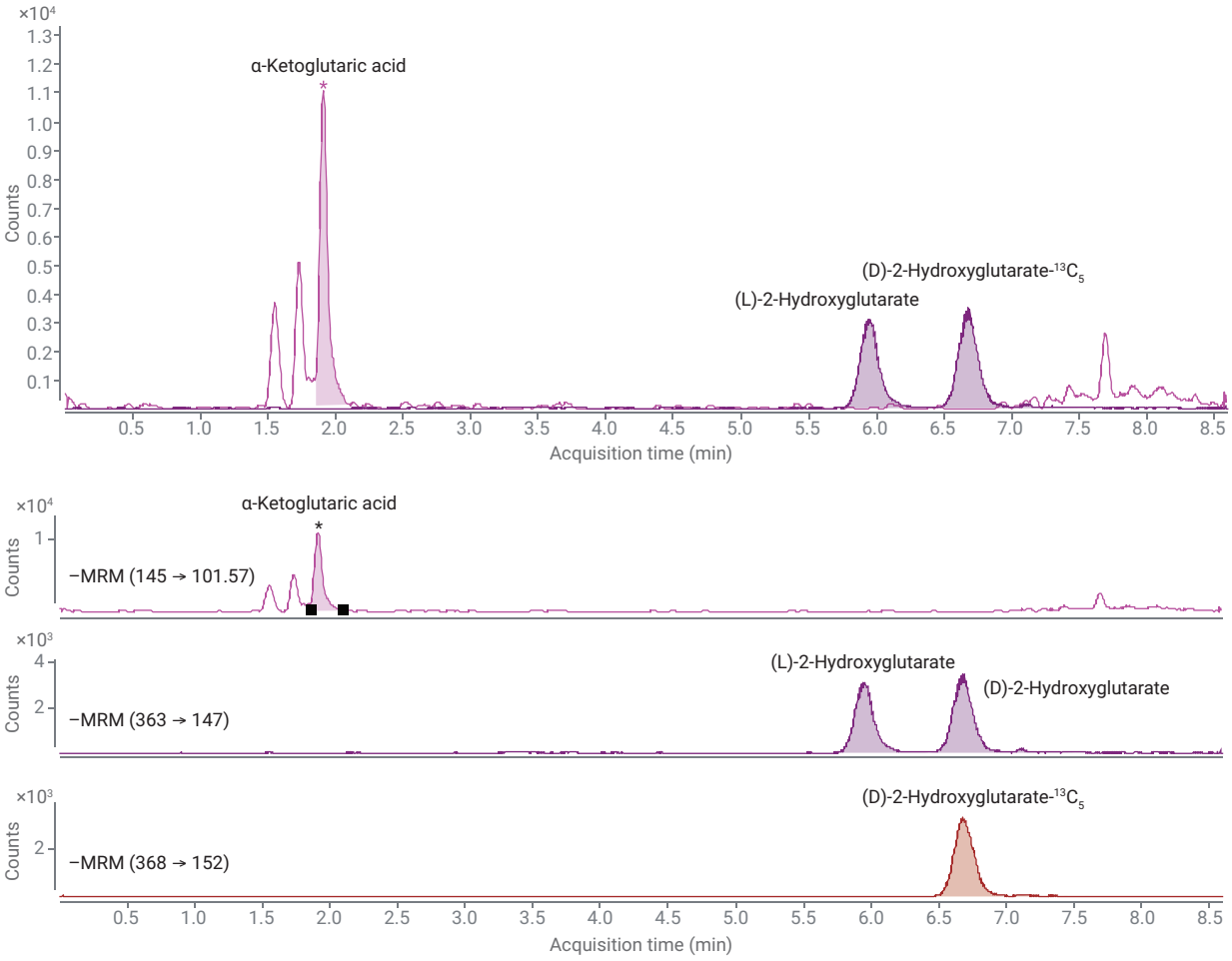

Figure 3. MRM chromatograms representing the separation of (D) and (L)2-HGA, D-2-HGA-$^{13}C_5$, and αKG in serum samples.

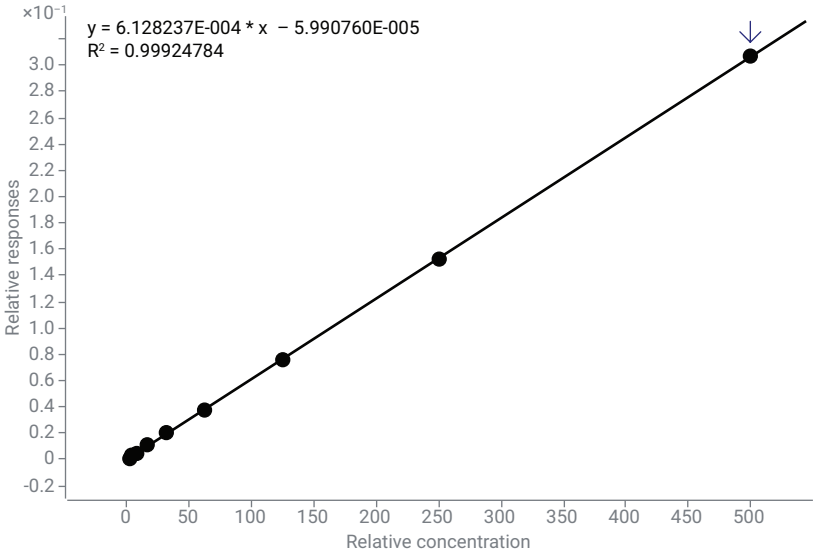

Figure 4. Calibration curve of αKG.

When evaluating the precision of this assay, the approach for estimating repeatability for any given level was to perform five replicate analyses in a single run on a single day. Precision was <15.

The linear calibration curve was not forced to zero. For both compounds, a calibration function was determined by linear regression over 0.4–120 µM with acceptable coefficient of determination.

Conclusion

This single LC/MS/MS method, developed after a derivatization procedure with DATAN, allows the quantification of both (L) and (D)2-HGA and αKG without derivatization in biological fluids (serum and plasma). This method can be used for clinical research as a tool and predictive biomarker for translational research. The results generated can be used to calculate the ratio of 2-HGA relative to the amount of αKG present, which will relate to the phenotype. Our LC/MS/MS method showed rapid separation with good sensitivity for the determination of both enantiomers without a chiral column. In addition, the LOQ values obtained were comparable to those obtained using previously described methods.

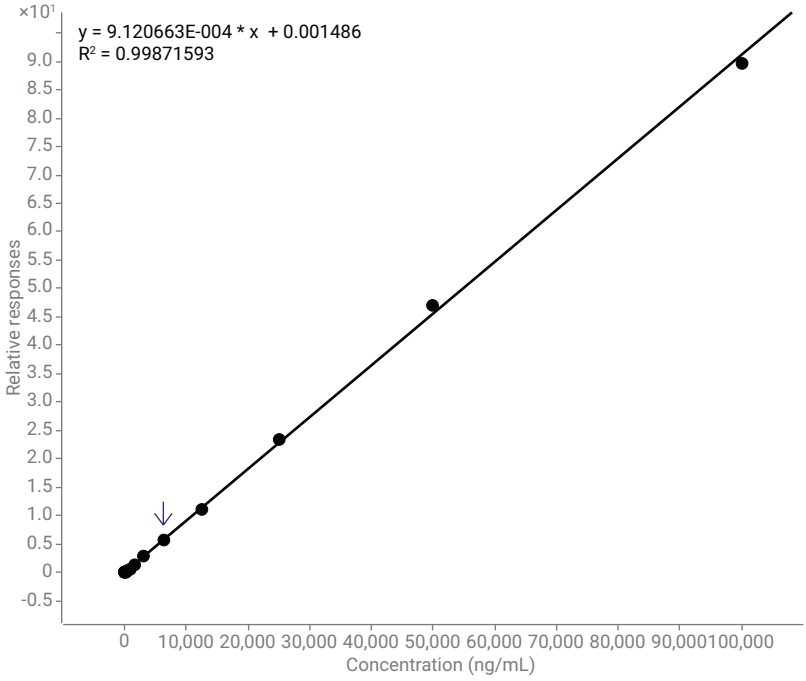

Figure 5. Calibration curve of 2-HGA.

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