**Separation and quantification of serum L- and D-2-hydroxyglutarate enantiomers by LC-MS/MS following derivatisation**

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*Introduction*

The compound 2-hydroxyglutarate (2-HG; Figure 1) is normally present at low levels in cells and is interconverted to alpha-ketoglutarate by 2-HG dehydrogenase (Fathi *et al.*, 2012). 2-HG exists as two stereoisomers (L-2-HG and D-2-HG, or *(S)*-2-HG and (*R)*-2-HG respectively) due to an asymmetric carbon atom in the carbon backbone, and both are endogenous metabolites found at similar levels in normal individuals (Struys 2013, Janin *et al*, 2014).

Elevated 2-HG is found in the inherited metabolic disorder 2-hydroxyglutaric aciduria and either L- or D-2-HG is overproduced (or both) depending on the mutation. For diagnosis of the precise disorder quantification of the stereoisomers rather than total 2-HG is helpful (Struys *et al.*, 2004). In some forms of cancer such as acute myeloid leukaemia (AML), the enzyme isocitrate dehydrogenase (IDH) is mutated (this is found in 15-20% of AML patients), leading to an overproduction of D-2-HG. Elevated 2-HG in serum predicts the presence of the mutation, and quantification of the stereoisomers separately improves specificity (Janin *et al*, 2014). Serum 2-HG concentrations declined during treatment of AML patients (Fathi *et al.*, 2012) and therefore 2-HG could be used to monitor the response to chemotherapy. Monitoring of levels post-treatment is suggested to be a marker of prognosis (Janin *et al*, 2014).

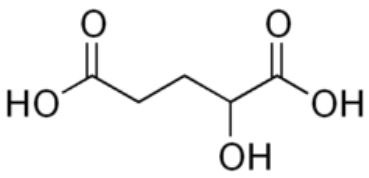


**Figure 1. Schematic of the structure of 2-HG**

Reliable quantification of the enantiomers separately is more technically challenging than measuring total 2-HG and requires the use of either a chiral separation column or a chiral derivatisation reagent (Struys *et al.*, 2004). We have used the derivatisation reagent DATAN (diacetyl-L-tartaric anhydride) to form diasteroisomers of L-2-HG and D-2-HG which we were then able to separate and quantify using an LC-MS/MS method.

*Methods*

Serum samples (25 µL) spiked with varying concentrations of L- and D-2-HG (Sigma) together with 25 µL of internal standard (20 mg/L of each of L- and D-2-HG-d3) were extracted by protein precipitation with 50 µL 0.1M zinc sulphate and the addition of 300 µL methanol. The supernatant was then dried down, and 100 µL of 50 mg/mL DATAN in a 4:1 solution of dichloromethane and acetic acid was added. The samples were heated to 75 °C for 30 minutes and were allowed to cool before drying and reconstitution with 100 µL water.

Using a Waters Acquity I Class UPLC, 10 µL of sample was injected onto a Waters Acquity UPLC HSS T3 1.8 µm, 2.1 x 50 mm column. A gradient elution was used with initial conditions of 95% mobile phase A (1.5 mM ammonium formate in water adjusted to pH 3.6 with formic acid) and 5% mobile phase B (methanol) for 1.2 minutes, stepping up to 95% mobile phase B for 0.5 minutes, before returning to initial conditions for 2.8 minutes (total run time was 4.5 minutes). A Waters Xevo TQ tandem mass-spectrometer was used and the specific transitions monitored were *m/z* 363>147 (L- and D-2-HG) and 366>150 (L- and D-2-HG-d3).

*Results*

Utilising samples spiked with just L- or D-2-HG, L-2-HG was found to elute first at approximately 1.1 minutes, with D-2-HG at 1.3 minutes. The derivatised stereoisomers were reproducibly separated and were quantified using the ratio of peak area to internal standard area and a standard curve. The assay was linear up to 250 µmol/L.

The intra-batch imprecision of the assay was found to be 2.6% (L-2-HG) and 5% (D-2-HG) at 50 µmol/L. Average recovery of 100 µmol/L of L-2-HG and D-2-HG spiked into serum samples was 88.15% and 89.45% for L- and D-2-HG respectively, compared to 100.0% (L-2-HG) and 96.5% (D-2-HG) for PBS/BSA spiked samples.

*Conclusions*

We have developed an assay for the separation and quantification of the two stereoisomers of 2-HG in serum using a small sample volume and with no need for a specialist chiral separation column. The derivatisation reaction is relatively quick and easy to perform in a routine laboratory environment. Measurement of both stereoisomers would be informative in the diagnosis of inherited metabolic disorders and in investigation and follow-up of patients with AML.

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*References*

Fathi AT, Sadrzadeh H, Borger DR *et al.,* (2012). Prospective serial evaluation of 2-hydroxyglutarate during treatment of newly diagnosed acute myeloid leukemia, to assess disease activity and therapeutic response. Blood 120(23):4649-4652.

Janin M, Mylonas E, Saada V *et al.,* (2014). Serum 2-hydroxyglutarate production in IDH1- and IDH2-mutated de novo acute myeloid leukemia: a study by the Acute Leukemia French Association group. J Clin Oncol 32(4):297-305.

Struys EA, Jansen EEW, Verhoeven NM, and Jakobs C (2004). Measurement of Urinary D- and L-2-Hydroxyglutarate Enantiomers by Stable-Isotope-Dilution Liquid Chromatography–Tandem Mass Spectrometry after Derivatization with Diacetyl-L-Tartaric Anhydride. Clinical Chemistry 50(8):1391–1395.

Struys EA (2013). 2-Hydroxyglutarate is not a metabolite; D-2-hydroxyglutarate and L-2-hydroxyglutarate are! PNAS 110(51):E4939.