HILIC/MS METHOD FOR THE QUANTIFICATION OF THE POLAR BIOMARKER ITACONIC ACID

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INTRODUCTION

Itaconic acid is a branched fatty acid which has been identified as a potential biomarker in alerting to a number of medical conditions including: gestational diabetes mellitus (GDM) and rheumatoid arthritis. Early detection and quantitation of this analyte in blood could enable earlier medical intervention and improve patient health.

Itaconic acid being a small polar molecule is not easily retained on reversed-phase liquid chromatography and has in the past been analysed using gas chromatography coupled to a mass spectrometer with a derivatisation step required in the sample preparation as well as by a number of ion-pair liquid chromatography methods. While both of these methodologies have proven to be successful they both have draw backs due to their laborious nature and the need for dedicated instruments.

Hydrophilic liquid chromatography (HILIC) has been shown to enable the retention and quantitation of highly polar compounds by utilising an aqueous layer on the surface of the analytical column which interacts with polar molecules and elutes analytes in order of increasing hydrophilicity.

In the work presented here, we describe a HILIC method using an 100mm x 2.1mm, sub 2 µm Amide column with a UHPLC instrument coupled to a triple quadrupole mass spectrometer. Itaconic acid was extracted from blood serum using an ion exchange solid phase extraction cartridge, in combination with a protein precipitation step, requiring no derivatisation prior to injection.

METHODS

LC-MS

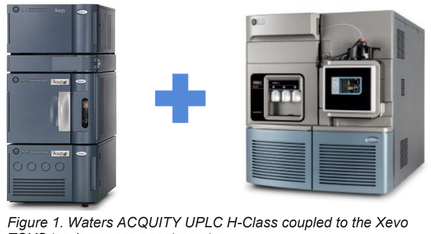


Figure 1. Waters ACQUITY UPLC H-Class coupled to the Xevo TQXS tandem mass spectrometer

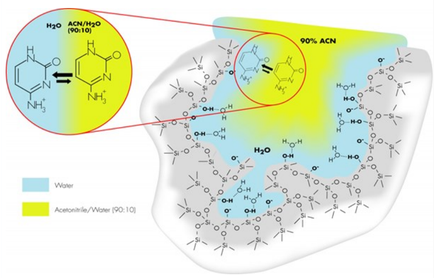
Parameter	Value		
Mobile Phase A (MPA)	90:10 ACN: 5mM Ammonium acetate (pH 9.0)		
Mobile Phase B (MPB)	50:50 ACN: 5mM Ammonium acetate (pH 9.0)		
Flow Rate	0.8mL/min		
Injection Volume	3µl		
Gradient Conditions	Time(mins)	MPA	MPB
	0	99	1
	0.87	99	1
	2.01	95	5
	2.67	48	52
	3.67	48	52
	3.8	99	1
	7.0	99	1
Column	ACQUITY BEH Amide 100 x 2.1mm, 1.7µm		
Column temp.	60°C		

Table1. UPLC conditions

5mM ammonium acetate at pH 9.0 was chosen for optimum peak shape/retention. Buffer is added to both A and B to ensure ionic strength is maintained throughout the gradient, preventing disruption of the aqueous layer on the HILIC column which would impact analyte peak shape/reproducibility (*Table 1*).

METHODS (CONTINUED)

WHAT IS HILIC?



Combination of partitioning, ion-exchange and hydrogen bonding

- Polar analyte **partitions** between bulk mobile phase and partially immobilized polar layer on material surface
- Secondary interactions between surface silanols and/or functional groups with the charged analyte leading to **ion-exchange**
- **Hydrogen bonding** between positively charged analyte and negatively charged surface silanols

- **HILIC - Hydrophilic Interaction Chromatography**
  - **Term coined in 1990 to distinguish from normal-phase[1]**
- **HILIC is a *variation* of normal-phase chromatography without the disadvantages of using solvents that are not miscible in water**
  - **"Reverse reversed-phase" or "aqueous normal-phase" chromatography**
- **Stationary phase is a POLAR material**
  - **Silica, hybrid, cyano, amino, diol, amide**
- **The mobile phase is highly organic (> 80% ACN) with a smaller amount of aqueous mobile phase**
  - **Water (or the polar solvent(s)) is the strong, eluting solvent**

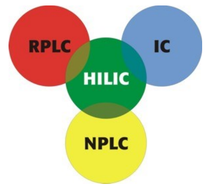


Figure 2. Overview of HILIC mechanism of action.

SAMPLE PREPARATION

1:1 PPT of 200µl serum with 0.1% TFA using Ostro™

Vortex well and centrifuge at 4000rpm for 15mins

Remove 400µl of supernatant and pre-treat with 4% H_3PO_4

Condition a Waters' Oasis™ WCX µElution plate with 200µl MeOH followed by equilibration using de-ionised water

Load all 800µl of the pre-treated samples to the wells

Collect flow through, vortex and inject directly

Figure 3. Overview of sample preparation method

Figure 3 gives an overview of the sample preparation protocol that was carried out to mitigate the itaconic acid binding to the matrix which had been established in the development process. Various pH values were looked at to determine the optimum value for dissociation of the itaconic acid from matrix proteins (*Figure 4*), along with a range of sample matrix : organic ratios to maximise recovery and reduce matrix effects.

Looking at *Figure 4*, 1.0% TFA would appear to be the best option according to recovery, however, sensitivity is a key goal in this assay and therefore, the 0.1% TFA modification is the best option.

SAMPLE PREPARATION (Continued)

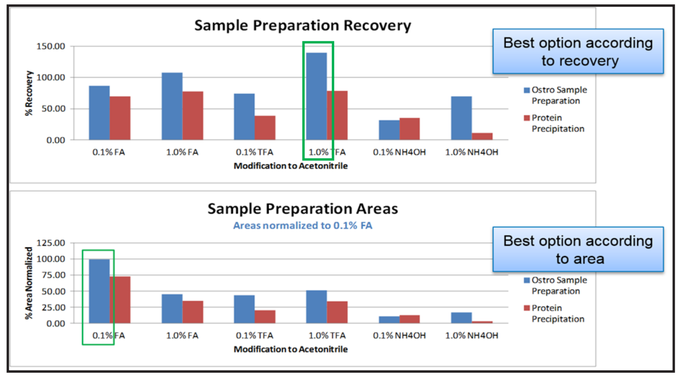


Figure 4. Optimisation of protein precipitation using a wide range of pH values. 1.0% trifluoroacetic acid (TFA) gives the best results according to recovery, whereas 0.1% TFA gives the best results according to area.

Further sample preparation optimisation was carried out to improve sample recovery (*Figure 6*). The ratio of organic to sample is also important with a 1:1 ratio giving the best recovery and minimal matrix effects. As human plasma samples are plentiful, 200µl can be used to maximise signal intensity of the endogenous itaconic acid.

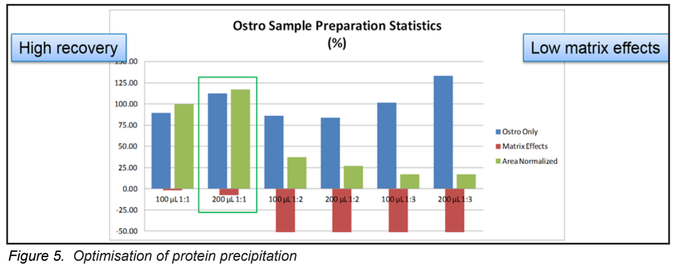


Figure 5. Optimisation of protein precipitation

As can be seen in *Figure 6* recovery results are normalised against the 0.1% TFA to ensure that signal intensity is optimised along with recovery and matrix effects.

RESULTS

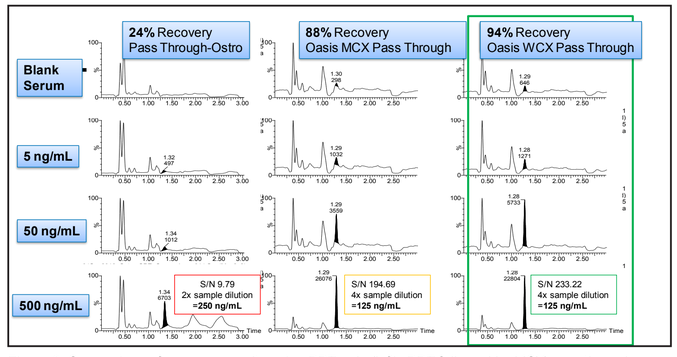


Figure 6. Comparison of recovery results using PPT only (left), PPT followed by MCX pass through (centre) and PPT followed by WCX (right)..

Using the protein precipitation conditions detailed in *Figures 5/6*, followed by a pass through sample preparation using a weak cation exchanger (WCX) gives the best results for recovery and signal intensity when compared to the mixed mode (MCX)cation exchanger and the protein precipitation only (*Figure 6*).

Linearity (R^2 0.997) was achieved over the range 2.5 - 500ng/mL with a mean accuracy range 88.7-110.8%. The biological relevance of itaconic acid is reported to be between 5.2 - 260ng/mL.[2]

RESULTS (CONTINUED)

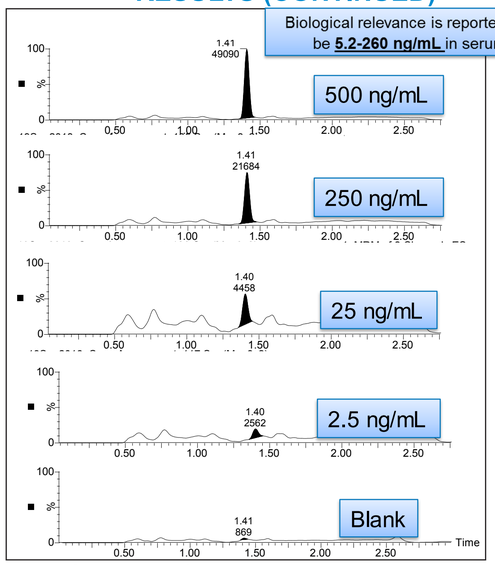


Figure 7. Chromatograms of spiked itaconic acid in plasma

	Linearity (r^2)	Dynamic Range* (ng/mL)	Weighting	Mean Accuracy Range (%)
Itaconic Acid	0.9971	2.5-500	1/x	88.7-110.8

Table 2. method used for system dispersion calculation

As this is a feasibility study there were no QC samples. For each calibration standard (n=3) mean accuracy was ± 12% satisfying the acceptance criteria (±15%), and for LLOQ (±20%).

CONCLUSION

- **A high end tandem quadrupole mass spectrometer can be successfully used to reliably and reproducibly quantify itaconic acid in human serum.**
- **Use of HILIC-MS with sub 2µm particle amide column provides sufficient retention, peak shape and sensitivity.**
- **A WCX SPE sample preparation was developed, which provides excellent recovery and selectivity, enabling LOD's ~ 1ng/mL (based on LOQ's), sufficiently detecting endogenous levels of itaconic acid.**
- **Excellent linearity (R^2 0.997) was achieved over the range**
- **Acceptable accuracy was achieved over the range 88.7-110.8) satisfying the acceptance criteria (±15%) and (±20%) for LLOQ**

FURTHER DEVELOPMENT

Further optimisation of this method planned:

- **Alternative MRM transitions to minimise endogenous interferences.**
- **Reduction of LC cycle time**
- **Adjustment of sample preparation method to minimise background interference**
- **Incorporate labelled, internal standard for added robustness**

References

1. Alpert, A.J.*J.Chromatography*, 499(1990) 177-196.
2. Targeted profiling of polar intracellular metabolites using ion-pair-high performance liquid chromatography and –ultra high performance iquid chromatography couples to tandem mass spectrometry: applications to serum, urine, and tissue extracts. Michopoulous, F., Whalley, N., Theodoridis, G., Wilson, I. D., Dunkley, T. P.J., Critchlow, S.

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