

# Determination of Methylmalonic Acid in Plasma using ZIC<sup>®</sup>-HILIC Chromatography and Single Stage Mass Spectrometric Detection

The detection and quantitation of Methylmalonic acid in plasma can be performed in approximately 3 min using a ZIC<sup>®</sup>-HILIC column with mass spectrometric detection. The assay requires simple protein precipitation and succinic acid does not interfere. The limit of detection is 10 nM and limit of quantitation is 30 nM.

## Introduction

Determination of Methylmalonic acid (MMA), Figure 1, in serum, plasma and urine is performed to monitor cobalamin (vitamin B<sub>12</sub>) deficiency. The assay is a functional marker of intracellular coalbumin status and is used for both diagnostic purposes as well as for monitoring treatment response.

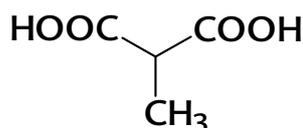


Figure 1: Structure of Methylmalonic Acid

Since MMA is present at low nanomolar levels in body fluids, is quite polar, has a low absorption coefficient in the UV region and is non volatile, current chromatographic methods for monitoring the compounds include extraction and derivatization followed by GC-MS or HPLC/CE methods with fluorescence detection. These approaches are expensive and time consuming and are the main reason why chromatographic approaches are not commonly performed. This technical note describes the detection of MMA in plasma samples using conditions that ensure that succinic acid [SA] (a structural isomer of succinic acid that is present at higher concentrations) does not interfere).

In recent years, the use of hydrophilic interaction liquid chromatography (HILIC) with mass spectroscopic detection has become a powerful approach for clinical analyses. HILIC is a very powerful technique for the separation of complex mixtures of polar compounds. It separates compounds using a mostly organic hydrophobic mobile phase with a hydrophilic stationary phase. The solutes elute in order of increasing hydrophilicity, which is the opposite of the elution order in reverse phase chromatography and is especially useful for the separation of polar compounds that are poorly separated by reverse phase.

ZIC<sup>®</sup>-HILIC chromatography is a unique form of HILIC that involves the bonding of zwitterionic sulfobetaine groups to a silica or polymer backbone of the stationary phase and thus allows for a significant aqueous fraction in the mobile phase. This allows greater solubility of polar analytes in the mobile phase and therefore provides greater sensitivity.

## Experimental

**Reagents:** MMA and succinic acid were obtained from Sigma-Aldrich Sweden AB. Acetonitrile (Lichrosolv HPLC grade and acetic acid (p.a.)) were obtained from Merck. Reagent grade water was obtained from a Milli-Q system (Millipore, Bedford MA). Deuterated MMA (Cambridge Isotope Laboratories) was used as the internal standard

**Samples:** Pooled plasma with normal endogenous MMA was obtained from a local blood bank and samples previously analyzed by GC-MS were obtained from the Bergen University Hospital. Samples were deproteinated with acetic acid and acetonitrile containing deuterated MMA.

**Instrumentation:** An Agilent 1100 LC/MSD with degasser, pump, autosampler, thermostatted sample compartment, ChemStation and a single quadrupole mass spectrometer was employed. MMA and the internal standard were monitored at *m/z* 117.2 and *m/z* 120.2 in negative ESI mode.

**Chromatographic Separation:** The separation was performed with a PEEK ZIC<sup>®</sup>-HILIC column (100 mm x 2.1 mm ID) packed with 3.5 μm, 100 Å particles (p/n 1.50441.0001, Merck KGaA, Darmstadt, Germany) at 30°C. An isocratic mobile phase of acetonitrile: 100 mM ammonium acetate (pH 4.5) was employed at a flow rate of 400 μL/min. 4 μL of precipitated plasma was injected. After separation, the column was washed for six minutes (acetonitrile: buffer 55:45 at a flow rate of 800 μL/min) and reconditioned for one minute.

## Results

Figure 2 presents the chromatogram collected at *m/z* 117.2. SA is neutral at pH 4.5 and elutes in the void volume.

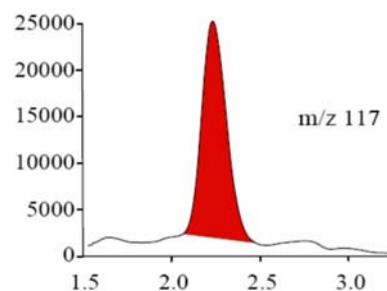


Figure 2: Separation of MMA - ZIC<sup>®</sup>-HILIC column. Mobile phase: CH<sub>3</sub>CN:100 mM ammonium acetate (pH 4.5).

Preliminary experiments indicated that the concentration of acetic acid and the volume ratio between the plasma sample and the precipitating solution were critical and a partial least squares analysis was performed. It was determined that a 3:1 ratio was optimum, but for routine studies, a 4:1 ratio was used to allow for the optimum column lifetime. Recoveries for MMA were found to be between 90 and 93% under these conditions. Within-day and between day CV's were better than 5% RSD.

The Limit of Quantitation (LOQ) for MMA was 30 nM (with a S/N of 10:1 and the Limit of Detection (LOD) was 10 nM (with a S/N of 3:1), estimated from patient samples containing less than 200 nM. Least squares regression analysis of the peak area ratios vs the analyte concentration indicated that the assay is linear from the LOD to 1000 nM (extended calibration curves up to 200  $\mu$ M demonstrates that the method is useful over at least four orders of magnitude).

Table 1 shows inter-day and intra-day data describing sample variability for controls and standards on 6 days of analysis over a 10 week period (n=18 except day 4 and 6).

	Control		C+100 nM		C+1000nM	
Day	Conc (nM)	S.D.	Conc (nM)	S.D.	Conc (nM)	S.D.
1	169.5	4.7	256.0	5.1	1104.6	23.6
2	179.8	5.7	264.0	7.9	1126.9	30.5
3	178.7	4.3	270.3	8.3	1144.7	52.9
4	177.8	10.7	277.7	10.1	1083.2	22.7
5	169.5	4.7	256.0	5.1	1104.6	23.6
6	179.8	3.4	289.7	8.7	1100.7	40.1
Avg.	175.8		269.0		1110.8	
%RSD	2.6		3.1		1.9	

The data from the method described herein was compared to an established GC-MS method with 67 samples and good agreement was obtained as shown in Figure 3.

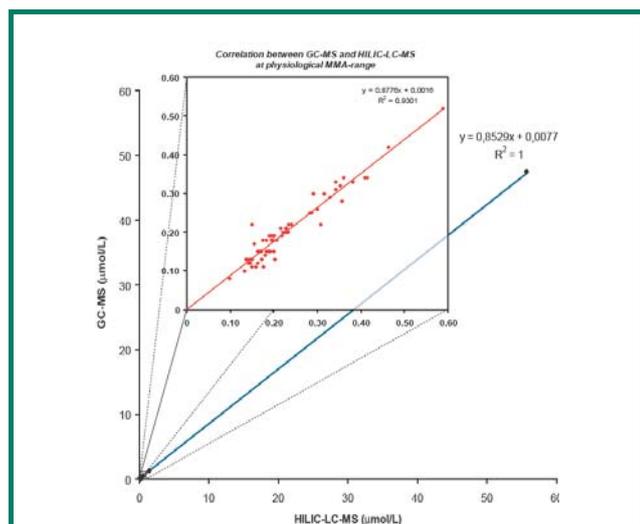


Figure 3: Linear correlation plot between a GC-MS method for MMA and the ZIC®-HILIC-MS method described herein. The insert shows the same measurements after exclusion of the two highest MMA values.

## Conclusions

The assay described herein allows for rapid, direct determination of MMA at physiological levels in plasma or serum. It does not require derivatization of MMA and is capable of distinguishing between the compound of interest and SA, which is typically present at higher concentration than MMA. MMA and SA are exceedingly difficult to separate via reverse phase HPLC, but are readily separated by the ZIC®-HILIC column. While ion suppression can be a serious problem when a single stage mass spectrometer is employed, this was not an issue in this assay; this may be due to the selectivity provided by the ZIC®-HILIC column.

The assay can be performed in about ten minutes and is very robust. If desired, sample throughput could be significantly increased via column switching for the column washing step and theoretically more than 400 samples could be performed per day.

## About ZIC®-HILIC Chromatography

The ZIC®-HILIC stationary phase is based on the covalently bonded permanent zwitterionic sulfobetaine group indicated in Figure 4. It is available with a silica support in 3.5, 5 and 10  $\mu$ m particle sizes in various column dimensions from capillary to semi-preparative (75  $\mu$ m up to 20 mm ID). In addition, it is available with a polymeric support on 5  $\mu$ m particles (ZIC®- $\mu$ HILIC). Merck SeQuant also publish the tutorial booklet [A Practical Guide to HILIC](#), which is available free of charge.

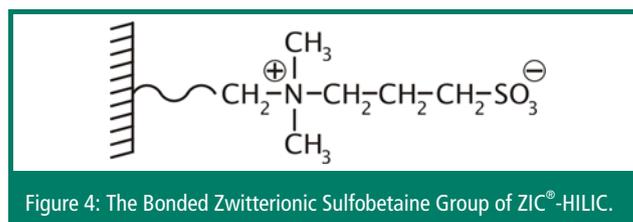


Figure 4: The Bonded Zwitterionic Sulfobetaine Group of ZIC®-HILIC.

## About Merck SeQuant

Merck SeQuant is a Swedish company that develops a broad range of innovative products for separation and purification of complex samples via chromatography. The company was founded in 1987 by researchers from Umeå University. Products include ZIC®-HILIC columns for HPLC, Membrane Suppressors and Suppressor Regeneration Systems for Ion Chromatography, Proteomic Calibration Kits, Post Column Reaction Delay and Mixing Coils which are marketed and supported around the world by a series of distributors.

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## References

Note: This application note is condensed from the scientific paper "Quantification of Methylmalonic Acid in Human Plasma with Hydrophilic Interaction Liquid Chromatography Separation and Mass Spectrometric Detection" Hans-Åke Lakso, Patrik Appelblad and Jörn Schneede. Clin. Chem., Oct 2008; doi:10.1373/clinchem.2007.101253