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AS116

Wellbrook Court I Girton Road I Cambridge I CB3 0NA I
+44 (0) 1223 279210 I fax: +44 (0) 1223 279253
enquiries@anatune.co.uk I anatune.co.uk

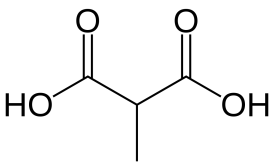
AUTOMATING THE ANALYSIS OF METHYLMALONIC ACID IN PLASMA AND SERUM USING A GERSTEL MULTI PURPOSE SAMPLER COUPLED TO LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY DETECTION

Paul H. Roberts[1], Renata Gorska[2] & Dr Dominic Harrington[2]. Anatune Ltd. Girton, Cambridgeshire, UK[1] & GSTS Pathology LLP. St Thomas' Hospital, Westminster Bridge Road, London, UK[2].

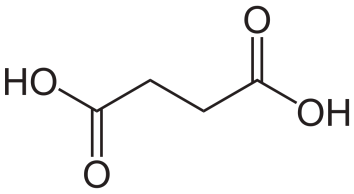
INTRODUCTION

Measurement of methylmalonic acid (MMA) in serum or plasma is useful for diagnosing cobalamin deficiency. Methylmalonic acid (MMA) determination in serum or plasma was introduced as a marker of cobalamin (vitamin B12) deficiency more than 20 years ago. Separation of MMA on reversed-phase liquid chromatography columns is difficult because MMA shows poor retention. The MMA structural isomer succinic acid (SA) may

also cause interference. Concentrations of SA in the serum or plasma are usually considerably higher than for MMA. Recently, the combination of hydrophilic interaction liquid chromatography (HILIC) with mass spectrometry has gained interest. In particular, the bonded zwitterionic stationary-phase ZIC-HILIC chromatography medium (Merck SeQuant) which is suitable for separating polar compounds such as MMA. Hydrophilic and weak electrostatic interactions between the stationary phase and MMA allow separation in the presence of high concentrations of organic solvent.



MMA Structure



Succinic Acid Structure

Presented is methodology outlining fully automated sample preparation and analysis to quantify MMA in serum or plasma that combines HILIC separation with tandem mass spectrometry analysis. Sample preparation is fully automated and consists only of protein precipitation and centrifugation.

INSTRUMENTATION

GERSTEL MPS 2, fitted with 250 µl syringe and LC injection valve
Anatune CF-200 centrifuge
Maestro Version 1.4.12.14
Agilent 6430 Triple Quadrupole Mass Spectrometer with HotBox and electrospray source.
Agilent 1200 Series HPLC
- G1312B Binary Pump SL
- G1316B Thermostatted Column Compartment SL
- G1379B Degasser

Figure 1: Anatune CF200 centrifuge

METHODOLOGY

Three sets of calibrators were prepared. Firstly in LC/MS grade water, secondly in charcoal stripped, mixed gender, pooled human serum and thirdly in EDTA treated, mixed gender, pooled human plasma. See Table 1. These calibrators were prepared from a super stock solution (8468 µmol/L) of MMA in methanol. This solution was further diluted in water to an intermediate concentration (50.8 µmol/L) and then diluted further in water, serum or plasma to form the calibrants.

For sample preparation, 200 µl of serum or plasma is placed into a 2 ml glass screw top autosampler vial and the vial capped using a magnetically transportable PolyMag™ caps (GERSTEL, Germany). The sample is then placed on the vial tray of the multi purpose sampler (MPS). See Figure 2. The following aspects of sample preparation are fully automated, conducted via the MPS and the CF-200.

Table 1: Levels of MMA in the three sets of calibrants

Cal Level	MMA nmol/L
Std_01	54
Std_02	108
Std_03	270
Std_04	540
Std_05	1080
Std_06	2700
Std_07	5400

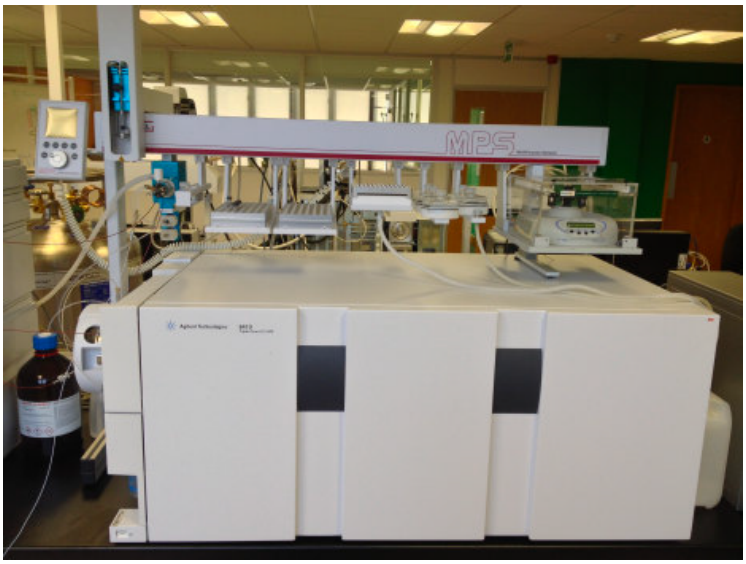


Figure 2: GERSTEL MPS for automated MMA extraction.

Extraction of MMA from serum or plasma is achieved through a simple protein precipitation. 800 µl of acetonitrile containing 0.5 % acetic acid and the internal standard MMA-d3 at a concentration of 156 nmol/L is added to the sample. The vial is then moved using magnetic transport to the CF-200 centrifuge whereby the contents are thoroughly vortexed for 30 seconds to assist in the protein precipitation. The vial is then centrifuged at 3000 rpm for 1 minute to separate the proteins from the supernatant in preparation for injection.

Sample analysis is fully automated by means of an external injection valve and loop fitted onto the MPS, 20 µl of extract is injected. Separation is achieved by means of an Merck PEEK ZIC®-HILIC 2.1 I.D. x 100 mm; 3.5 µm particle size column. The chromatographic mobile phases consisted of acetonitrile (eluent A) and 100mM ammonium acetate adjusted to pH 4.5 with formic acid in water (eluent B). An isocratic elution followed by a gradient column reconditioning wash was performed, see table 2. LC run time was 8 minutes, column temperature was maintained at 30 °C. the presence of high concentrations of organic solvent

Table 2: LC gradient conditions

Time	% A	% B	Flow ul/min
0.00	70	30	400
2.40	70	30	400
2.41	40	60	800
5.00	40	60	800
5.01	70	30	800
7.00	70	30	800
7.01	70	30	400
8.00	70	30	400

An Agilent 6430B tandem mass spectrometer with electrospray source was used in negative ionization mode. Instrument analysis time was 8 minutes per sample using the conditions listed in table 3. Column effluent was diverted to waste for the first 0.8 minutes of the analysis and then again to waste at 2.4 minutes for the remainder of the run to ensure the MS source remained clean.

Table 3: Selected MS conditions for analysis.

Parameter	MMA	MMA-d3	Parameter
Precursor ion	117	120	Precursor ion
Product	73	76	Product
Product ion (q)	55	58	Product ion (q)
Dwell	50	50	Dwell
Fragmentor (V)	60	60	Fragmentor (V)
Collision Energy Q (V)	5	5	Collision Energy Q (V)
Collision Energy q (V) 25 25	25	25	Collision Energy q (V) 25 25
Cell Acc. (V)	5	5	Cell Acc. (V)

Gas Temp (°C): 350 Gas Flow (l/min): 5 EMV: -900V
Nebulizer (psi): 50 Capillary (v): 2500

RESULTS

Calibration curves were constructed for MMA in water, serum and plasma. Linear calibrations for all matrices were achieved from the seven point calibration standards. Correlation coefficients of 0.998, 0.998 and 0.997 were achieved for water, serum and plasma calibrations respectively. See figures 3,4 and 5.

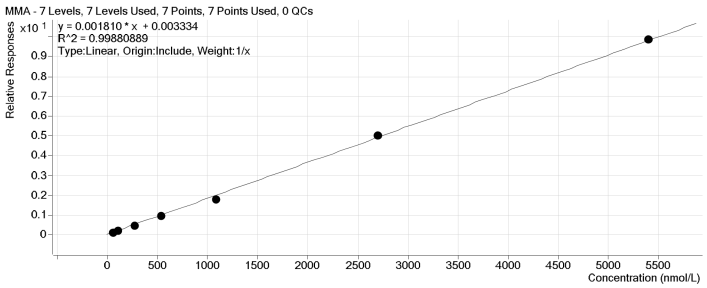


Figure 3: Linearity of water based standards

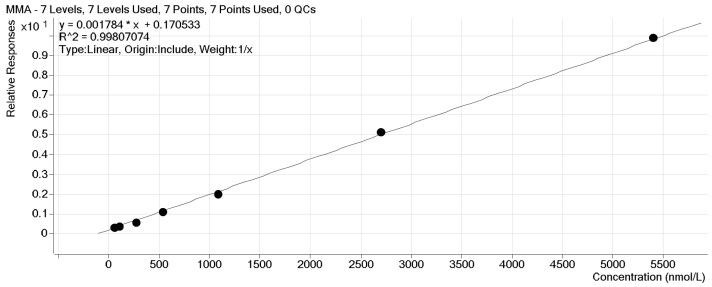


Figure 4: Linearity of serum based standards

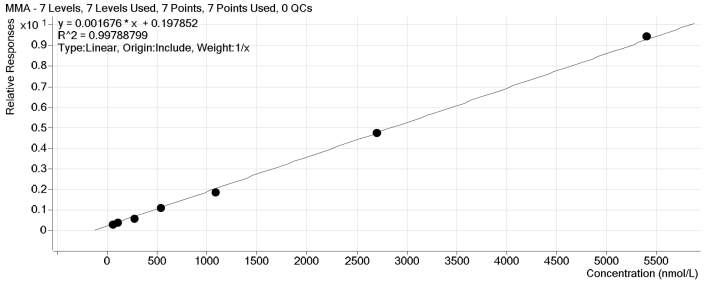


Figure 5: Linearity of plasma based standards.

The accuracy and precision of the method were determined for MMA in both serum and plasma using samples of low and middle concentrations (108 & 540 nmol/L). Table 4 shows the resulting accuracy and precision data for both matrices. For MMA in serum, accuracy data averaged 101 % (range 99.9 – 102.7 %) and precision data averaged 1.1 % RSD (range 0.4 - 1.8 %). For MMA in plasma, accuracy data averaged 99.8 % (range 99.6 – 99.9 %) and precision data averaged 0.8 % RSD (range 0.6 - 1.0 %).

Table 4: Accuracy and precision data

Matrix	QC 108 nmol/L	QC 540 nmol/L
	Serum	
Mean	110.9	539.6
SD	2	1.9
% RSD	1.8	0.4
Ave. % Accuracy	102.7	99.9
	Plasma	
Mean	107.9	537.9
SD	1.1	3
% RSD	1	0.6
Ave. % Accuracy	99.9	99.6

Extraction recovery for MMA in serum and plasma was assessed by comparing the average peak area ratios from the extracted midpoint concentration samples (540 nmol/L) in serum and plasma to the average peak area ratios of water based standards prepared at an equivalent concentration. As shown in Table 5, the recovery for MMA in serum was found to be 118 % and in plasma 115 %.

Ion suppression effects were monitored by assessing the change in response of MMA and MMA-d3 internal standard in the calibration batches of water, serum and plasma matrix. There were no signs of ion suppression for MMA and MMA-d3 despite the very simple sample preparation.

One possible explanation is that serum and plasma samples were diluted with 800 ul of acidified acetonitrile, which could have influenced the matrix/analyte ratio positively, essentially helping to dilute out the matrix. Furthermore, the absence of ion suppression could be due to the selectivity afforded by the HILIC column. At pH 4.5, the majority of matrix constituents may either be uncharged or show low retention; however, other compounds may be strongly retained and could possibly be eluted during the column washing step.

Table 4: Accuracy and precision data.

Matrix	Water	Serum	Plasma
Response (MMA/ISTD)	0.96088	1.13253	1.10212
	0.97623	1.13853	1.09071
	0.9509	1.13374	1.10172
	0.96954	1.12908	1.10099
	0.93875	1.13324	1.10245
Average	0.95926	1.13342	1.0996
SD	0.015	0.003	0.005
% RSD	1.55	0.3	0.45
% Recovery		118	115

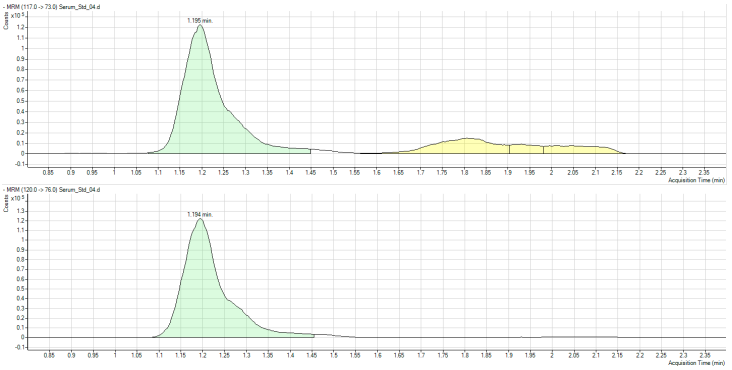


Figure 6: Below shows a typical chromatogram from a 540 nmol/L serum calibration standard.

CONCLUSIONS

Presented is a fully automated method for MMA analysis featuring automated internal standard and reagent addition, protein precipitation and centrifugation. Sample preparation is coupled directly to LC-MS/MS and is fully integrated within the MassHunter software. Sample preparation takes place during the preceding sample analysis for optimal system throughput and utilisation.

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REFERENCES

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