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Semi-automated quantification of methylmalonic acid in human serum by LC-MS/MS

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ABSTRACT

Background Methylmalonic acid (MMA), a sensitive biomarker of functional vitamin B12 deficiency, is commonly determined by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods using manual extraction and derivatization of MMA to reduce polarity prior to separation.

Methods In the present study we introduce a semi-automated extraction on a strong anion exchanger, HPLC separation on a BEH-amide column to separate serum MMA from its abundant isoform, succinic acid, followed by MS/MS detection and quantification.

Results The extraction of MMA plus internal standard provides full recovery and the method is linear between 0.03µmol/L and 20.0 µmol/L (r^2=1.0) with intra-and inter-assay imprecision of 2.2%. Agreement with other laboratories has been demonstrated in external proficiency testing. Compared to both conventional GC-MS and LC-MS/MS methods, the correlation is r^2> 0.99.

Conclusions The use of robotic pipetting, elimination of derivatization and improved separation by the BEH-amide column combined with HILIC chromatographic conditions significantly improve sample throughput compared to conventional methods. Using a single pipetting robot and LC-MS/MS instrument, this method is currently performing 180 analyses per day from10 regional hospitals and several additional distant sites.

Keywords: Chromatography, High Performance Liquid, Tandem Mass Spectrometry, Gas Chromatography-Mass Spectrometry, Methylmalonic acid, Vitamin B12.

Introduction

Vitamin B12 (cobalamin) plays an important role in the formation of red blood cells and in the maintenance of neuronal function [1]. Its deficiency can lead to a wide spectrum of hematological and neuro-psychological disorders that can be reversed by early diagnosis and prompt treatment [1, 2]. As the conversion of methylmalonyl-coenzyme A (CoA) to succinyl-CoA is a vitamin B12 dependent reaction in humans [3, 4], an elevated plasma level of methylmalonic acid (MMA) is a sensitive biomarker of vitamin B12 deficiency [5-7], more accurately reflecting functional vitamin B12 deficiency than plasma B12 concentration itself [7, 8]. Large clinical studies have identified a 2.5th-97.5th percentile reference limit of MMA for middle-aged subjects (age 47-49 years) of 0.10-0.28 µmol/L serum and for elderly subjects (71-74 years) of 0.10-0.36 µmol/L [6, 9]. Pregnant women have had mean MMA 0.10 µmol/L (95% CI 0.10-0.11), newborns had 0.27 (0.26-0.28) and 6 month old infants 0.52 (0.46-0.59) µmol/L, respectively [10].

Different techniques, most frequently including GC-MS, capillary electrophoresis and high pressure liquid chromatography (HPLC) coupled to tandem mass spectrometry (LC-MS/MS), have been used to measure MMA in clinical applications [11-14]. Conventional GC-MS methods require laborious extraction and derivatization to a non-polar form prior to a slow reverse-phase chromatographic separation to resolve structurally related compounds. Although LC-MS/MS is rapidly replacing GC-MS in clinical laboratories due to its relative methodological simplicity [13-17] most current LC-MS/MS methods for MMA determination still use derivatization [13-16] and few utilize automation. Our goal was to simplify and automate preanalytical handling and to speed separation to increase throughput, while retaining accuracy and precision in a routine clinical setting.

Analytical methods

Materials and samples

A homogeneous pool of sera from 200 healthy volunteers was available at the laboratory and used as matrix for calibrator solutions. Anonymized normal and patient serum samples from the Department of Clinical Chemistry, Helsingborg Hospital, Sweden, collected in SST tubes (Becton Dickinson) and centrifuged at 3000 rpm (1770 g) for 10 min. and saved as aliquots have been used for method development and quality control. Two MMA serum controls with concentrations of 0.21µmol/L and 0.74µmol/L are from SERO, Norway. Purified MMA (Sigma-Aldrich) and tri-deuterated methyl-malonic acid (MMA-d_3), from Cambridge Isotope Laboratories Inc., are used as calibrator and internal standard, respectively. Solid-phase strong anion-exchange (SAX-SPE) (Isolute®) columns in 96-well format, containing 25 mg of resin with 1 mL reservoirs and 96-well sample collecting plates are from Biotage, Sweden. All other reagents are of highest available grade and water is deionized and millipore filtered in all cases.

Preparations of calibrators and internal standard

Five calibrators are prepared according to standard addition technique by adding purified MMA corresponding to (Cal 1, 0.00; Cal 2, 0.10; Cal 3, 0.40; Cal 4, 1.00µmol/L and Cal 5, 4.00µmol/L) to homogeneous pooled serum from 200 healthy volunteers. A 1.00 mmol/L stock solution of internal standard MMA-*d3* (IS) is prepared by dissolving 12.1 mg MMA-*d3* in 100 mL 20% methanol and diluting to a 0.50 µmol/L working solution in water. Aliquots of calibrators and the internal standard are stored at -70 ºC for up to 12 months. Thawed aliquots of both are stored at + 4 ºC for up to 2 weeks prior to use.

Extraction of MMA and MMA-d3 from serum

The entire sample handling procedure, from sample application, to dissolution of extracted MMA and MMA-*d3* is performed by a robotic pipetting system (Fredom EVO, TECAN) at

room temperature, with barcode labeling of samples and plates to ensure sample tracking. 150µL sample is mixed well with 300µL MMA-*d3* working solution in a 0.80 mL 96-well plate. A 300µL portion of each sample is robotically pipetted to a strong solid-phase anion-exchanger (SAX-SPE) (Isolute®) that has been pre-wetted with 0.50 mL methanol followed by 0.50 mL water for 5 min each. MMA and MMA-*d3* bound on the strong anion exchanger is washed first with 0.50 mL water followed by 0.50 mL methanol for 5 min each, and then eluted using 600µL eluting solution (3% (v/v) formic acid in acetonitrile) into a 96-well sample collecting plate with vacuum at 0.05 bars under the plate. The elution solution is then evaporated to dryness by air flow at 50 L/min at 50 ºC for 15 min in a SPE Dry96 instrument (Biotage, Sweden), and the residue is dissolved in 150µL HPLC mobile phase (100% acetonitrile: 100mmol/L ammonium acetate, pH 4.5; 80/20; v/v). Barcode labeling of primary sample tubes and 96-well plates ensures sample tracking.

Quantitative mass spectrometric analysis

An 8µL aliquot of each sample is automatically injected from the 96-well sample collecting plate into the HPLC Acquity system with a BEH-amide column (No. 186004800, 2.1 mm x 50 mm, 1.7 µm particle size, Waters) and separation of MMA and succinic acid is achieved in a mobile phase composed of solvent A (100% acetonitrile) and solvent B (100 mmol/L ammonium acetate, adjusted to pH 4.5 with formic acid) under a gradient process, as shown in Table I. MMA and MMA-*d3* are passed essentially simultaneously at ca 0.80 min. followed by succinic acid at 1.16 min. to a Waters Quatro Micro tandem mass spectrometer equipped with the atmospheric pressure ionization (API) probe operating in negative mode. The following conditions are used: capillary voltage 0.7kV, cone voltage 14V, source and API probe temperatures of 150 and 350 ^{o}C, respectively. Desolvation nitrogen flow is set to 700L/h. For MS/MS analysis, argon is used as collision gas with a cell pressure of 4.5 x 10^{-3} mbar. Energy of collision is set to 9eV. The transitions monitored are *m/z* 117 → 73 for

detection of MMA and *m/z* 120 → 76 for detection of MMA-*d3*. Control of all system components and data acquisition is performed in multiple reaction-monitoring (MRM) modes with MassLynx 4.1 Software. Data processing and quantification of MMA are performed by the QuanLynx Application Manager (Waters Inc.).

Method validation

The five calibrator solutions and two serum controls are included in each analytical run. Recovery has been assessed by addition of 0.50 and 1.00 µmol/L pure MMA together with the standard volume of IS to duplicate serum samples prior to extraction on the SAX-SPE columns and following extraction but prior to evaporation of the sample. Linearity is demonstrated by serial dilution of the serum pool following addition of 21.6 µmol/L pure MMA. Intra-assay imprecision has been determined for 2 clinical samples with concentrations 0.24 and 0.95 µmol/L MMA (N=10) and inter-assay imprecision is calculated from results of two commercial MMA controls with concentrations 0.21 µmol/L and 0.74 µmol/L. Results for clinical samples in a concentration range from 0.1 to 2.5 µmol/L are compared to those for the same samples analyzed by conventional LC-MS/MS [14] and GC-MS [11] methods, following sample derivatization. Accuracy is finally evaluated by proficiency testing by the Danish Institute for External Quality Assurance for Laboratories in Health Care (DEKS) in comparison to results from 37 – 40 other Scandinavian laboratories.

Pre-analytical stability of serum samples has been evaluated on 33 serum samples stored at +4^{o}C from 8 hrs to 5 days after sample collection and centrifugation. Results using initial serum sample volumes of 100µL, 160µL and 320 µL, containing a constant volume of internal standard working solution have been compared.

Statistical analyses

Comparisons of pre-analytical stability and of results using different serum volumes and dilutions as well as comparisons between alternative methods have been calculated using

linear regression analysis. Internal and external control statistics are reported as CV%. Statistical analyses have been performed with the statistical software GraphPad Prism 5 (La Jolla, CA, USA). Calculations of number of theoretical plates and resolution of separation use IUPAC standard equations (http://old.iupac.org/publications/books/author/mcnaught.html).

Results

Analytical characteristics

The typical peaks of MMA, MMA-*d3* and succinic acid are shown in Figure 1, using the transitions *m/z* 117 → 72 and *m/z* 120 → 75 to monitor the peaks of MMA and MMA-*d3*, respectively. The retention times of MMA and MMA-*d3* are about 0.80 min, with no interference from the MMA isomer, succinic acid that has retention time about 1.16 min. Calculations from typical analyses demonstrate the number of theoretical plates to be 625 for MMA and 5330 for succinic acid with a resolution of 3,25 for the separation. During method development, MMA levels in serum, EDTA- and heparin- plasma were determined, with no systematic differences in results (data not shown).

Method validation

Linearity: Calibration curves are consistently linear with MMA (µmol/L) = 1.28 X AUC (95% CI: 1.25 – 1.31) + 0.007 (95% CI: -0.001 – 0.015), and r^2 = 1.00 (95% CI: 0.999 – 1.00) (N = 20). Quantification is based on comparison of the peak areas for MMA and MMA *d3* (IS) fragments. At 0.1 µmol/L the signal to noise ratio was 10. This was not investigated at lower concentrations. The recovery of MMA, based on raw AUC data was 86.4% at MMA concentration of 0.80 µmol/L and 98.3% at 1.30 µmol/L. Recovery of the IS in the same samples was 86.4% and 97.7% respectively, providing a calculated recovery in MMA concentration of 100.0 % and 100.3%, respectively. Linearity in serial dilutions using either pooled serum or water demonstrate linearity between 0.03 µmol/L and 22 µmol/L with

measured value = 1.01 expected value – 0.02 µmol/L (Figure 2A) and the absence of significant matrix effects. Although the initial result exceeded the maximal calibrator, a single patient sample gave an initial MMA concentration of 25.47 µmol/L. Results of analysis of serial dilutions of this sample compared to expected values (y=1.004x-0,100, R2 = 1.00) verified linearity and absence of matrix effects over this broad concentration range. Analysis of a reagent blank following this and other elevated samples demonstrated < 0.1% carryover.

Imprecision: Intra-assay imprecision of patient samples (N=10) is 2.0% at 0.24 µmol/L and 2.2% at 0.95 µmol/L concentrations, with inter-assay imprecision for commercial MMA serum controls (N= 56) of 5.2% at 0.21 µmol/L and 2.2% at 0.74 µmol/L.

Accuracy: The analytical results of 44 samples using this method compared to the GC-MS method produce a linear regression curve y =0.96x + 0.03 (r^2=0.99) (Fig. 2B). Compared to an alternative LC-MS/MS method (Figure 2C, n=30), the regression equation is y=0.99x + 0.01 (r^2=0.99). The difference between our result and the mean value from > 36 other laboratories within the DEKS proficiency testing program for multiple samples with mean concentrations from 0.14 to 1.25 µmol/L has been -0.025 to 0.045 (µmol/L), and always < 2 SD from the mean (Figure 3). In the present method, the low background contributes also to the accuracy of the measurements. Analysis of samples containing from 0.1 to 0.5 µmol/L MMA are stable during storage at 4 ºC for 5 days Y=0.99X, R2 = 0.99). And no significant differences in results are seen after dilution of samples with initial volumes $\geq$ 100 µL to 150 µL with 0.9% saline solution, or use of 100 µL, 160 µL or 320 µL serum samples. 150µL serum has been selected for the standard protocol.

Discussion

Despite significant interest and high demand for MMA determination in clinical chemistry, its application in most clinical laboratories is still a challenge due to the low endogenous

concentration of MMA and potential interference from its isomer, succinic acid. The latter is considerably more abundant than MMA in human serum and difficult to remove. For many years, the GC-MS method has been considered as a golden standard for MMA determination [18]. This method, however, requires laborious sample preparation including extraction and derivatization steps, frequently with overnight incubation, followed by more than10 minute chromatographic separations per sample to resolve structurally related compounds. During recent years, LC-MS/MS has been rapidly replacing GC-MS in clinical laboratories due to its relative methodological simplicity [13-15].

This new method provides both accurate and precise determination of MMA in serum and plasma samples. The correlations of our results with other methods as well as accumulated results of clinical samples justify the use of published data [6, 9] concerning a normal reference interval of < 0.28 μmol/L for people < 50 years old and < 0.36 μmol/L for those ≥ 50 years old.

The use of the SPE-SAX extraction procedure with no chemical derivatition reduced the total pre-analytical sample preparation time to about 45 minutes, with the possibility to handle multiple batches of 90 samples, controls and calibrators in parallel on a single pipetting robot. Automated robotic pipetting into 96-well plates allows total sample tracking throughout the analytical process. The background level of our method is reproducibly low, contributing to linearity over a broad concentration range, as seen by accurate estimation at 25 μmol/L of one patient sample, verified by serial dilution. Due to characteristics of the BEH-amide column under HILIC conditions, high pressures can be used during the chromatographic step, reducing the actual instrument time to <3 minutes/sample, with total resolution (R_s = 3.25) of MMA and succinic acid peaks (Figure 1). High pressure chromatography and efficient

washing cycles prolong column life in this robust separation. Comparable results are obtained using 100µL, 160µL and 320µL sample volumes. The detection limit and linear range are highly relevant for samples within the normal reference intervals. MMA levels exceeding 4µmol/L, frequently present in pernicious anemia and primary methylmalonic acidemia, are indicated in the primary run and can be verified by analysis of diluted samples. The clinical need for analytical sensitivity far below the normal reference interval is not evident. Nonetheless, further sensitivity (allowing smaller sample volumes) can potentially be obtained by reducing the volume of mobile phase used for dissolution of the purified sample residue. This new method, using a single pipetting robot and one LC-MS/MS instrument, has now been in use for more than one year, frequently analyzing 180 samples per workday from 10 regional hospitals and additional external institutions. This can be compared to a previous 6-day workload for 200 samples.

In conclusion, automated robotic pipetting and the SAX-strong anion exchanger for extraction of MMA before HPLC separation on a BEH-amide column, provide successful separation of serum MMA from its abundant isoform, succinic acid. The current method reduces the time for sample preparation and chromatographic separation compared to conventional GC-MS and LC-MS/MS methods. Together these characteristics provide a high-throughput, precise, accurate and stable analytical method for MMA, fully suitable for clinical laboratories.

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Table I. Running procedure of LC-MS/MS

Running time (min)	Flow rate (ml/min)	Solvent A (%)	Solvent B (%)
Initial-1.60	0.40	80	20
1.61-1.90	0.60	55	45
1.91-3.00	0.40	80	20

Solvent A consists of 100% acetonitrile and solvent B consists of 100 mmol/L ammonium acetate, adjusted to pH 4.5 with formic acid.

Legend to figures

Figure 1. Typical chromatography of MMA, MMA-*d3* and succinic acid.

MMA-*d3* produces a peak with retention time 0.79 min monitored at *m/z* 120 → 75 for its fragment. MMA and succinic acid are clearly separated with retention times of 0.80 and 1.15 min. monitored at *m/z* 117 → 72 for its fragment. The calculated number of theoretical plates for this separation is N = 625 for MMA and 5330 for succinic acid, with resolution of separation Rs=3.25 (http://old.iupac.org/publications/books/author/mcnaught.html).

Figure 2. Validation of our LC-MS/MS method with GC-MS and an alternative LC-MS/MS.

A: Correlation between measured MMA and added MMA under serial dilutions in a serum pool.

B: Comparison of this LC-MS/MS to a GC-MS method (n=44).

C: Comparison of this LC-MS/MS to an independent LC-MS/MS method (n=30).

Figure 3. Results in external proficiency testing

Discrepancies from the results of this LC-MS/MS method (Hbg) from the mean of 37-40 laboratories (external control) for 8 control materials having mean concentrations from 0.14 to 1.25µmol/L. All differences are $\leq$ 0.045 µmol/L) and within $\pm$ 2 SD. The linear regression curve of our results vs. mean results is y =0.98x + 0.001 (r^2=0.99).

Figure 1

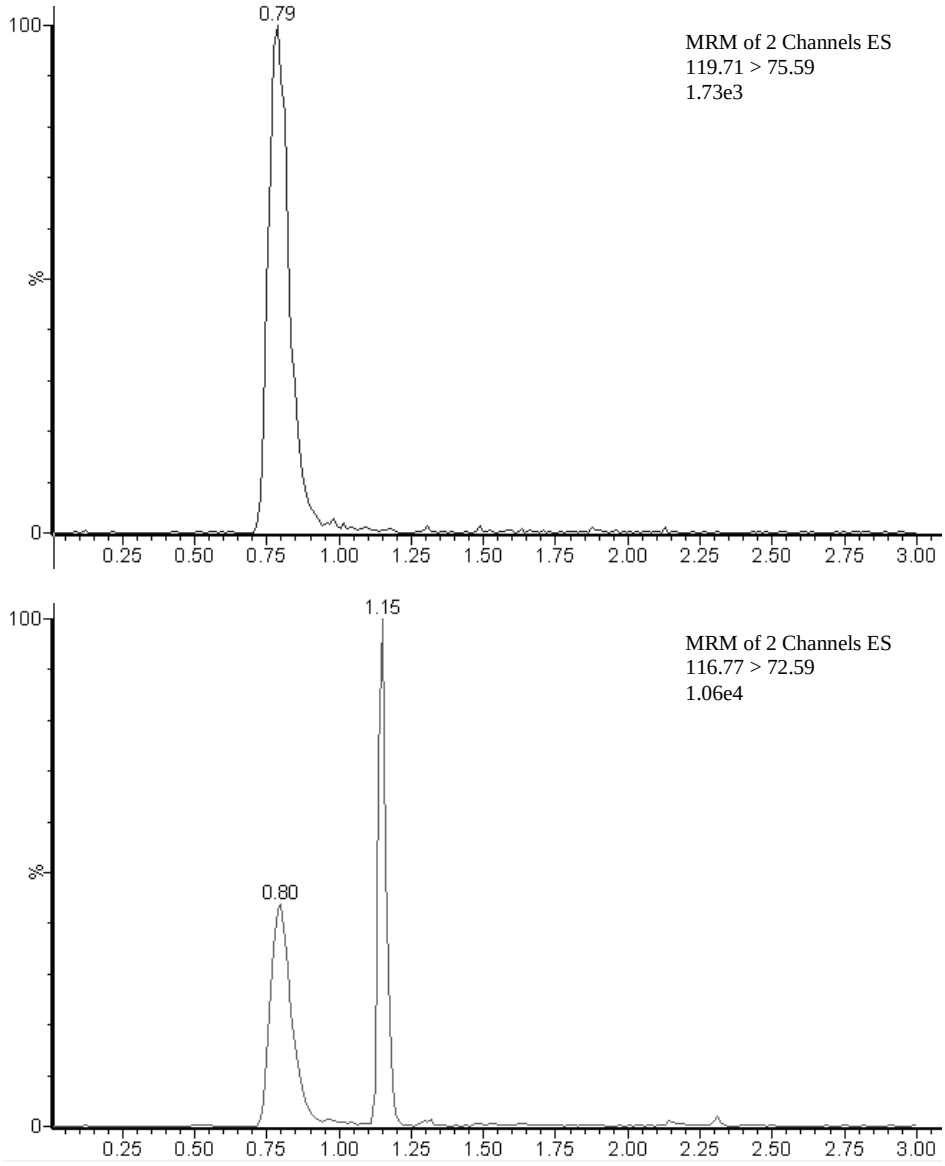

0.79
MRM of 2 Channels ES
119.71 > 75.59
1.73e3
100
%
0
0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75 3.00
1.15
0.80
MRM of 2 Channels ES
116.77 > 72.59
1.06e4
100
%
0
0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75 3.00

Figure 2

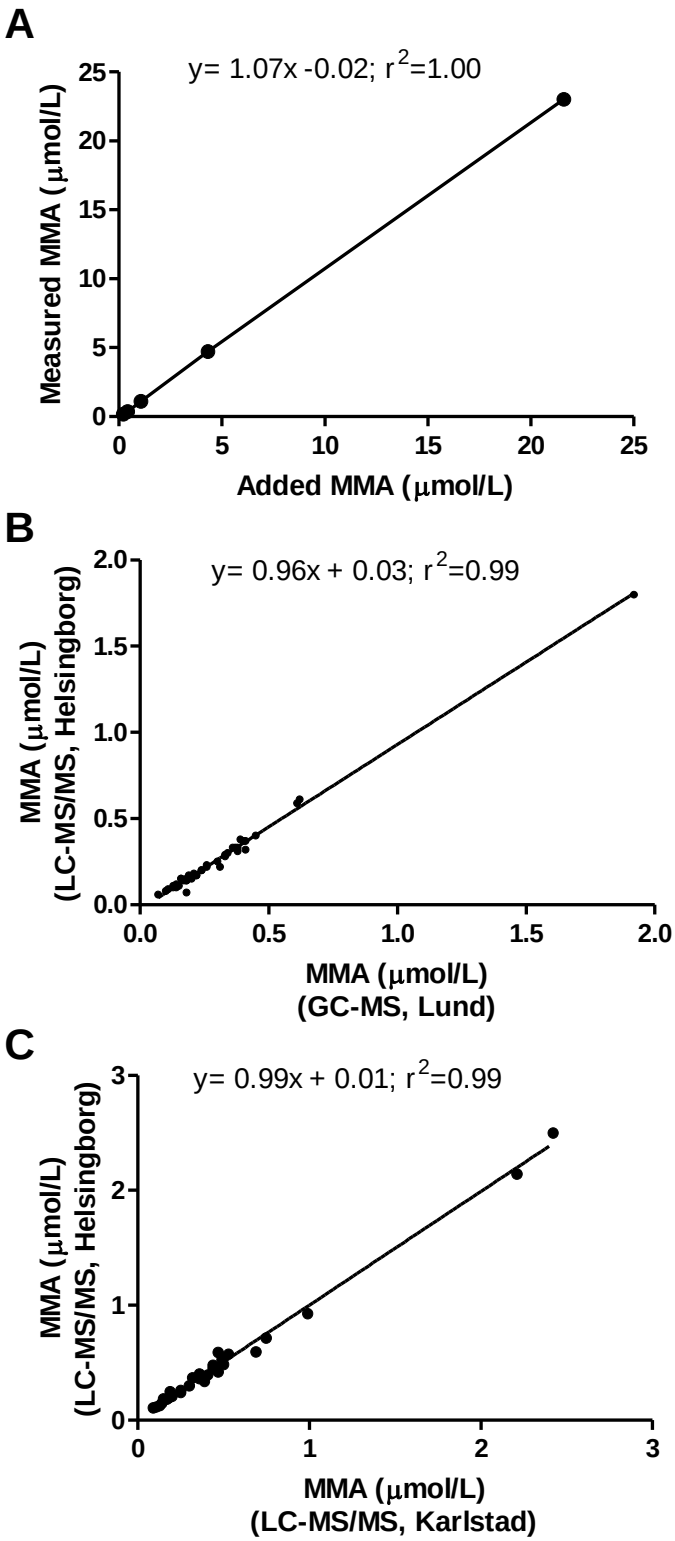
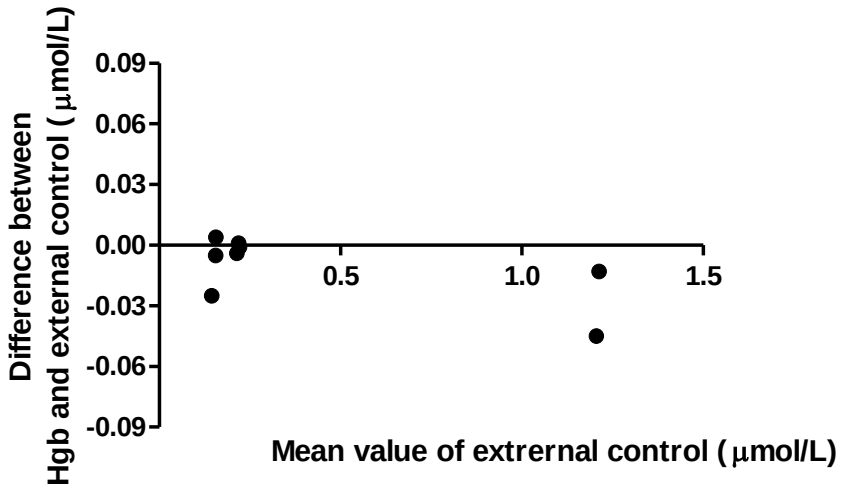

A
y= 1.07x -0.02; r2=1.00
Measured MMA (µmol/L)
Added MMA (µmol/L)
B
y= 0.96x + 0.03; r2=0.99
MMA (µmol/L) (LC-MS/MS, Helsingborg)
MMA (µmol/L) (GC-MS, Lund)
C
y= 0.99x + 0.01; r2=0.99
MMA (µmol/L) (LC-MS/MS, Helsingborg)
MMA (µmol/L) (LC-MS/MS, Karlstad)

Figure 3


Difference between
Hgb and external control (μmol/L)
0.09
0.06
0.03
0.00
-0.03
-0.06
-0.09
0.5
1.0
1.5
Mean value of extrernal control (μmol/L)