Evaluation of Two Sample Preparation Methods, Precipitation-Derivatization and SPE, for Quantitative LC-MS Analysis of Methylmalonic Acid in Plasma for Clinical Research

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ABSTRACT

To evaluate two sample preparation methods, precipitation-derivatization method (APCI in positive mode), and SPE method (HESI in negative mode), for the quantitative LC/MS analysis of methylmalonic acid (MMA) in human plasma samples using a triple quadrupole mass spectrometer for use in clinical research.

INTRODUCTION

Liquid chromatography-mass spectrometry (LC-MS) analytical methods are widely used for analysis of methylmalonic acid (MMA) in clinical research laboratories. Simple sample preparation, low limits of quantitation and separation from the naturally occurring structural isomer succinic acid (SA) are among the analytical method requirements. Here we evaluated the performance of two sample preparation methods for LC-MS analysis that fulfilled all of these requirements for clinical research needs.
The SPE method (SPE) utilized new Thermo Scientific™ SOLAμ™ extraction plates which use small volumes of eluting solvent resulting in elimination of the evaporation and reconstitution steps needed in conventional SPE methods. The protein precipitation-derivatization method (Deriv) was optimized to improve efficacy and reproducibility of derivatization reaction using a new additive-catalyst.

MATERIALS AND METHODS

Calibrators and QC Samples

•1.0 mg/mL MMA and 100 µg/mL internal standards (d3-MMA) were purchased form Cerilliant (Round Rock, TX)
•Since analyte free matrix was not available calibration standards were prepared in aqueous solution with 2% acetonitrile.
•QC samples Level I and Level II were purchased from RECIPE Chemicals (Munich, Germany).
•Level 0 QC was prepared from pooled donor plasma samples.
•Concentrations of QC samples and calibrators are specified in Table 1.

Table 1. Concentrations of MMA in QC samples

Plasma Level 0 (Deriv/SPE)	RECIPE Level I	RECIPE Level II
81.7 / 110	260	583

Instrument

•Thermo Scientific™ Dionex™ UltiMate™ 3000 HPLC system
•Thermo Scientific™ OAS-3X00TXRS autosampler
•Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer

Data Analysis

Thermo Scientific™ TraceFinder™ software version 3.2 was used for data acquisition and data processing.

Precipitation-Derivatization Method

Sample Preparation

- Mix: 100 µL of plasma sample + 200 µL of mixture of acetonitrile:methanol (9:1, v:v) containing 250 nM internal standard (d3-MMA). Vortex, centrifuge
- Transfer 100 µl of supernatant to disposable culture tube containing 50 µl of 1M zinc sulfate
- Evaporate* to dryness at room temperature under stream of nitrogen. A gas drying unit is highly recommended for the nitrogen flow.
- Add 200 µL of 3N HCl in n-butanol to each sample tube, then again evaporate* under nitrogen to dryness at room temperature. (No reaction time is needed)
- Add 300 µL of 50% methanol to each sample, vortex thoroughly, centrifuge. Transfer supernatant into an HPLC vial with limited volume insert if needed.
- Inject 20 µL onto LC-MS system.
- *: Evaporation under elevated temperature will shorten the drying time

LC method

- Column: Thermo Scientific™ Accucore™ C8, 2.6 µm, 50 x 2.1 mm at ambient
- Mobile Phase A: 0.1% formic acid in water
- Mobile Phase B: 0.1% formic acid in methanol
- Isocratic separation at 63.5% Mobile Phase B
- Flow rate: 0.5 mL/min

Mass Spectrometry Method

- APCI in positive ionization mode
- SRM method with ion ratio confirmation (Table 2)

Table 2. SRM Experimental Parameters

Analyte	Precursor (*m/z*)	Product Ion (m/z)	Collision Energy (V)	Qualification / Qualification	Polarity
MMA	231.2	175.2	6	Qualifier	Positive
MMA	231.2	119.2	11	Quantifier	Positive
d3-MMA	234.2	178.2	6	Qualifier	Positive
d3-MMA	234.2	122.2	11	Quantifier	Positive

SPE Method

Sample Preparation

- Conditioning the Thermo Scientific™ SOLAμ WAX 2mg/1mL/96 well plate (P/N: 60209-005) with 100 µL methanol followed by 200 µL 5 mM ammonium acetate at pH 4 (adjusted with formic acid)
- Centrifuge the plasma samples to ensure smooth SPE flow without clogging
- Deliver 100uL of calibrator, QC, or plasma sample onto the well which containing 300 uL of 5 mM ammonium acetate at pH 4 (adjusted with formic acid) with 5 uM d3-MMA internal standard, aspirate and dispense one time using the same pipet tip for better mixing
- Wash well with 100 uL of water (HPLC grade)
- Elute 2x 35 uL of 1% NH3 into the collecting plate which containing 30 uL of 10% formic acid
- Shake the plate for 30 sec, or transfer eluent into an HPLC vial with limited volume insert if needed.
- Inject 15 µL for LC/MS analysis

LC Method

- Column: Thermo Scientific™ Accucore™ RP-MS, 2.6 µm, 100 x 2.1 mm at ambient
- Mobile Phase A: 0.4% formic acid in water
- Mobile Phase B: 0.1% formic acid in methanol
- Flow rate: 300 – 700 uL/min (LC gradient: Table 3)

Table 3. LC Gradient

Time (min)	Flow rate (mL/min)	%A	%B
0	0.3	98	2
1.19	0.3	98	2
1.2	0.5	98	2
1.49	0.5	98	2
1.5	0.5	60	40
1.74	0.5	60	40
1.75	0.5	98	2
1.85	0.7	98	2
2.5	0.7	98	2

Mass Spectrometry Method

- HESI in negative ionization mode
- SRM method with ion ratio confirmation (Table 4)

Table 4. SRM Experimental Parameters (SPE)

Analyte	Precursor (*m/z*)	Product Ion (m/z)	Collision Energy (V)	Qualification / Qualification	Polarity
MMA	117.1	55.3	25	Qualifier	Negative
MMA	117.1	73.3	10	Quantifier	Negative
d3-MMA	120.1	58.3	25	Qualifier	Negative
d3-MMA	120.1	76.3	10	Quantifier	Negative

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METHOD PERFORMANCE EVALUATION

Limit of quantitation (LOQ) were defined as the lowest concentrations that had back-calculated values within 20% of nominal and RSD for 5 replicates within 20%. Figure 1 shows chromatograms of the lowest calibration standards.

Method precision and accuracy was evaluated by analyzing replicates of each QC sample (n=4, 5) in three different days (Table 5 and Table 6) and by analyzing replicate injections of each calibration standard (Table 7).

Matrix effects and procedure recoveries

- **Absolute % recoveries:** Internal standard spiked into 10 (Deriv) or 8 (SPE) processed donor plasma samples. Absolute % recovery was calculated against the same concentration of internal standard spiked into solvent (n=3) (Table 8).
- **Spiked recovery**: 100 nM of MMA were spiked into 10 donor plasma samples. Un-spiked and spiked donor plasma samples were analyzed in triplicates. Relative and absolute % recoveries were calculated against data collected for spiked solvent (Table 9).

Reproducibility of derivatization reaction: %RSD of internal standard peak area in processed blank plasma samples from 10 donors (n=5) were calculated (Table 10). Reproducibility may be underestimated due to any possible matrix effects.

RESULTS

Table 5. Intra-assay precision and accuracy. Replicates of QC samples analyzed in 3 batches

Analyte	Method	Level 0	Level I	Level II
Norminal Conc. (nM)		81.7 / 110	260	583
%RSD	Deriv. (n=5)	2.14 - 5.42	1.36 - 2.63	1.81 - 2.36
%RSD	SPE (n=4)	2.45 - 5.10	0.81 - 1.99	0.35 - 0.93
% Recovery	Deriv. (n=5)	92.5 - 97.6	86.8 - 89.9	94.1 - 94.6
% Recovery	SPE (n=4)	89.7 - 101	91.8 - 95.3	96.9 - 104

Table 6. Inter-assay precision and accuracy. Replicates of QC samples analyzed in 3 batches

Analyte	Method	Level 0	Level I	Level II
Norminal Conc. (nM)		81.7 / 110	260	583
%RSD	Deriv. (n=5)	4.6	2.4	2.0
%RSD	SPE (n=4)	6.4	2.2	3.5
% Recovery	Deriv. (n=5)	95.7	88.5	94.4
% Recovery	SPE (n=4)	96.7	93.1	99.7

Table 7. Replicate injections of each calibration standard-precision and accuracy

	Method	Cal1	Cal2	Cal3	Cal4	Cal5	Cal6	Cal7	Cal8	Cal9	Cal10
Conc. (nM)		25	50	100	500	1000	2000	10000	25000	50000	100000
%RSD	Deriv. (n=5)	2.82	4.14	2.06	2.59	3.9	---*	2.24	2.04	2.77	3.1
%RSD	SPE (n=4)	3.57	2.08	0.89	0.76	0.9	0.46	0.08	0.32	0.2	0.5
Avg. % Rec	Deriv. (n=5)	113	101	95.2	93.9	96.5	---*	94.8	105	102	98.2
Avg. % Rec	SPE (n=4)	109	102	105	94.5	94.1	95.1	99.5	101	99.9	100

*: This concentration was not evaluated in derivatization method.

Figure 1. Calibration curves (25 nM – 100,000 nM) and lowest calibration standard peaks for quantifying and qualifying product ions (a: Deriv. method, b: SPE method)

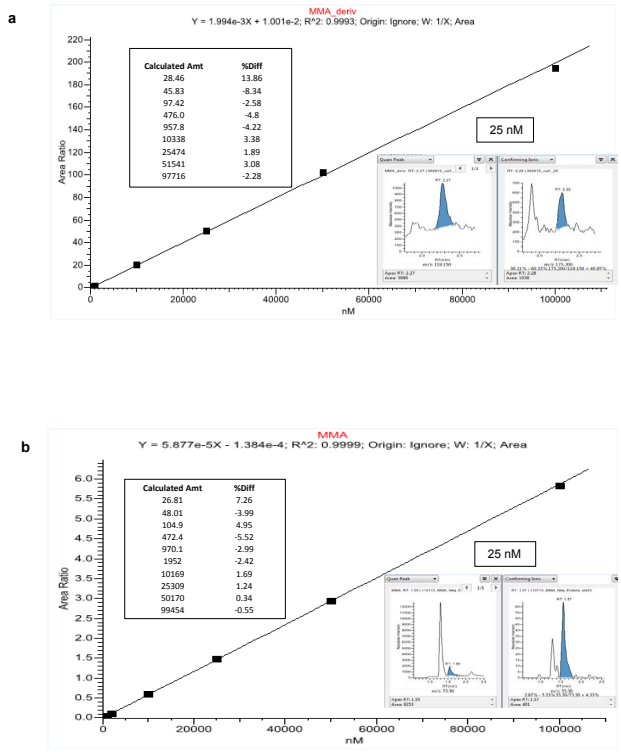


Figure 2. Chromatogram of quantifying ion and confirming ion at LOQ concentration (a: deriv. method, b: SPE method)

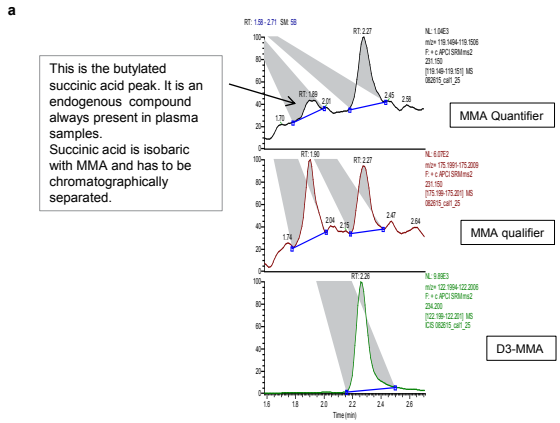


Figure 2. Chromatogram of quantifying ion and confirming ion at LOQ concentration (a: deriv. method, b: SPE method)

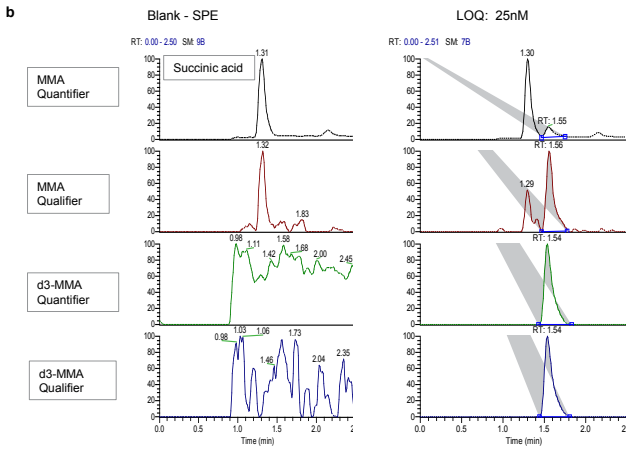
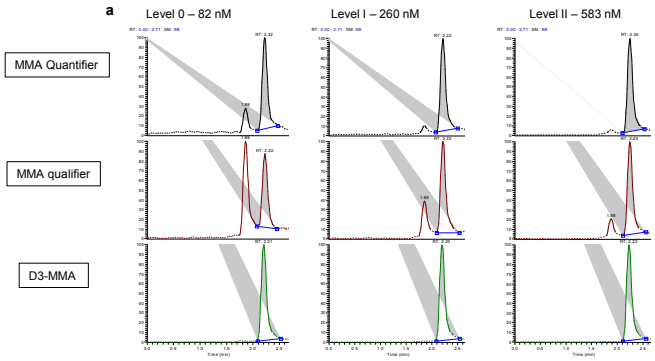


Figure 3. Chromatogram of quality control samples (a: deriv. method, b: SPE method)



b

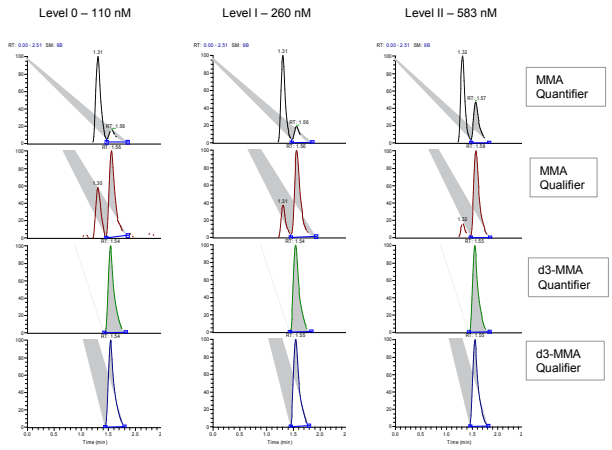


Table 8. Matrix effect: Internal standard absolute % recovery compared to samples prepared in solvent (10 donors for deriv. method and 8 donors for SPE method)

	Method	Plasma1	Plasma2	Plasma3	Plasma4	Plasma5	Plasma6	Plasma7	Plasma8	Plasma9	Plasma10
%RSD	Deriv. (n=3)	2.11	12.6	10.7	1.97	5.07	12.2	1.46	5.50	0.69	0.49
%RSD	SPE (n=4)	2.80	0.85	2.82	2.01	4.71	2.39	4.39	2.53	---	---
%Rec	Deriv. (n=3)	98.3	85.4	90.8	103	100	101	83.9	103	101	98.0
%Rec	SPE (n=4)	93.8	104	107	104	102	98.5	101	89.7	---	---

Table 9. Matrix effect/Spike recovery: Previously analyzed plasma from 10 donors were spiked with 100 nM of MMA

	Method	Plasma1	Plasma2	Plasma3	Plasma4	Plasma5	Plasma6	Plasma7	Plasma8	Plasma9	Plasma10
%RSD	Deriv. (n=3)	1.83	3.08	2.82	0.69	2.55	1.17	3.38	1.36	1.35	2.49
%RSD	SPE (n=4)	1.66	1.30	1.53	2.35	2.01	1.14	0.55	2.31	0.61	1.60
%Rec	Deriv. (n=3)	97.2	94.7	95.9	95.1	94.3	89.9	93.9	92.5	92.0	96.8
%Rec	SPE (n=4)	104	107	99.7	106	96.8	104	100	105	100	99.7

Table 10. MMA derivatization reaction process reproducibility

Method	Analyte	# of Donor Samples	# of Replicates	%RSD
Deriv. method	d3-MMA	10	n = 5	12.3

CONCLUSIONS

•Both sample preparation methods allow for LC-MS analytical performance meeting clinical lab requirements.
•Both sample preparation methods can be automated.
•The use of the newly discovered additive-catalyst is critical in the MMA derivatization method. It is proven to be highly effective in approving derivatization reaction completion, and consequently, the reproducibility of the derivatization reaction itself (without internal standard correction) is achievable (it is not achievable for most published MMA derivatization methods).
•Selection of specific sample preparation method method by the lab will depend on other then analytical performance factors e.g. consumables cost or sample preparation workflows already used in the lab.

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