JITMM Proceedings

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J I T M M Proceedings 7 (2018) 7-13

DETERMINATION OF ORGANIC ACIDS PRODUCED BY *PLASMODIUM FALCIPARUM* USING LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY

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ABSTRACT

Apart from lactic acid (LA), several recent studies indicated that p-hydroxyphenyllactic acid (pHPLA), α-hydroxybutyric acid (aHBA), and β-hydroxybutyric acid (bHBA) also play an important role in severe malaria cases with acidosis. This study aimed to determine the source of these organic acids, focusing on parasite-released products. *Plasmodium falciparum* samples were synchronized to yield a high percentage of ring stage sample. Supernatants from malaria culture were collected from 4 stages--ring form, trophozoite, schizont, and post-schizont rupture then, purified by solid phase extraction (SPE). Liquid chromatography – mass spectrometry (LC-MS) assay was develop to detect and quantitate the concentration of target analytes. LA was found to be a *P. falciparum* product at different concentrations in each stage, while pHPLA, aHBA, and bHBA were not detectable at significant levels using this specific LC-MS method. Amino acid depletion of culture medium and acid stability should be further investigated to achieve other aspect for future studies.

Keywords: *P. falciparum*, Organic acids, Acidosis, LC-MS, Malaria culture

INTRODUCTION

Lactic acid is generally measured in patients with metabolic acidosis and known as a major product released from parasite after glucose consumption through glycolysis pathway to meet its energy requirement during asexual erythrocytic stage. A study (Sriboonvorakul N, et al., 2013) was a preliminary to assess the organic acids in human plasma and urine which were hypothesized to be potential contributors of metabolic acidosis in sever malaria cases by development and validation of a novel liquid chromatographic - mass spectrometric method (LC-MS). These candidate acids were L-lactic acid (LA), α-hydroxybutyric acid (aHBA), β-hydroxybutyric acid (bHBA), *p*-hydroxyphenyllactic acid (pHPLA), malonic acid (MA), methylmalonic acid (MMA), ethylmalonic acid (EMA), and α-ketoglutaric acid (aKGA). Further study (Herdman MT, et al., 2015) characterized the role of these acids in severe malaria cases compared with uncomplicated malaria cases and other references. The result showed acid concentration in plasma and urine which indicated that only 4 from 8 acids include L-lactic acid (LA), *p*-hydroxyphenyllactic acid

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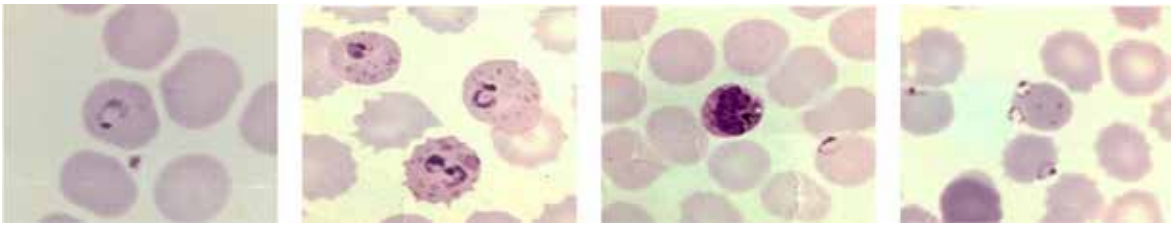
(pHPLA), α-hydroxybutyric acid (aHBA), and β-hydroxybutyric acid (bHBA) are elevated statistically significant (all $p<0.05$) in severe malaria cases compared with uncomplicated malaria cases. For this study, we chose these 4 acids to further investigate their source by focusing on parasite-released products *in vitro* and using healthy red blood cell as controls so, the objective was to measure these 4 organic acids which were found to play an important role in severe cases with acidosis according to the finding of study in 2015, and attempted to indicate whether those organic acids are directly produced by parasite.

MATERIALS AND METHODS

Sample collection: after synchronization, the culture with high percentage of predominant ring stage was adjusted to 1% P, 5% Hct, and separated into 2 parallel lines within 50 mL tissue culture flasks (Falcon 3014™, Becton Dickinson, USA) for carrying and collecting the supernatant from 4 different stages as presented in Figure 1. The culture medium was not changed. 20 mL total volume of culture started at 1% P, 5% Hct with ring stage was incubated at 37°C, 5% CO_2 for 6 hours. After that, mixed well and collected 4 mL to centrifuge at 800 x g, 4°C for 5 minutes before collecting supernatant. The supernatants were filtrated through 0.22-micron syringe filter then centrifuged again at 1,200 x g, 4°C for 5 minutes and stored at -80°C until analysis. For 16 mL remains in the culture flasks were re-incubated at 37°C, 5% CO_2 until the culture achieved high percentage of the interesting stage, then the samples were collected as the same procedure of collecting from ring stage. The control samples were from cultivation of normal red blood cells from healthy blood donors (O^+ cells) that was parallelly cultivated and collected at the same time with TM267. All the samples were sent to Clinical Pharmacology Laboratories, Mahidol - Oxford Research Unit and collected at -80°C until analysis by LC-MS method.

Sample preparation: Pre-treat sample was done by loading 100 μL of samples (supernatant) into 96-well plate followed by 100 μL of combined internal standard solution (IS solution) using an Eppendorf (Hamburg, Germany) stream multi-stepper. Then, added ammonium acetate buffer (pH 8.0; 2.5 mM) 800 μL with a 12-channel pipette, and mixed well at 600 rpm for 10 minutes on an Eppendorf MixMate™ (Hamburg, Germany). After that, centrifuged the 96-well plate at 1,100 x g for 10 minutes. Next, were the procedures of conditioning ISOLUTE PE-AX 96-well SPE plate (Biotage, Uppsala, Sweden) in order to prepare the plate for loading sample, 12-channel pipette was used throughout these procedures. The first step, added each empty SPE well with methanol 1 mL, and slowly applied vacuum about 3 - 5 ``Hg until there were no any residuals in well (this step was done twice). Then, added formic acid/methanol (15:85 v/v) to each SPE well as conditioning solution and slowly applied vacuum about 3 - 5 ``Hg for conditioning SPE to properly bind with acidic analytes and elute some impurities. Then, added ammonium acetate buffer (pH 8.0; 2.5 mM) 1 mL to each well and applied vacuum about 3 - 5 ``Hg until all wells were empty. Loading, 1 mL of



(a) (b) (c) (d)

Fig 1- Asexual stage of *P. falciparum* strain TM267 under microscope. (a) Ring form, (b) Trophozoite, (c) Schizont, (d) Post-schizont rupture.

pre-treated samples was loaded into conditioned SPE well plate and slowly applied vacuum about 1 - 1.5 ``Hg approximately 2 minutes. Escalated the vacuum about 0.5 ``Hg every 2 minutes until there were no samples remained in the wells. Washing, added water 1 mL followed by methanol 1 mL to each well and slowly applied vacuum about 3 - 5 ``Hg. After that, applied full vacuum around 10 minutes before drying the column tips with tissue paper. Elution, inserted collection plate into the vacuum manifold then added 950 $\mu$L formic acid/methanol (15:85, v/v) as the elution solution to each well. Slowly applied vacuum about 0.5 - 1 ``Hg for 2 minutes and escalated by 0.5 - 1 ``Hg every 2 minutes until there were no elution solvent in the plate. This is important to make sure that all the samples were slowly drained from the cartridge. Using a TurboVap (Caliper, Massachusetts, USA) with nitrogen gas at 40 ºC for eluents in collection plate in order to vaporize for dryness (2 hours). Reconstitution, added mobile phase (20 mM ammonium acetate - acetonitrile (40:60, v/v), pH 4.7) 200 μL using a multi-stepper auto pipette, and mixed on MixMate™ at 800 rpm for about 10 minutes. Lastly, 3 μL of the reconstituted extracts were injected in to the LC-MS system.

Instrumentation for liquid chromatography - mass spectrometry: The reagents and buffers used for analysis as HPLC / MS water, HPLC / MS Acetonitrile, and HPLC methanol were from J.T. Baker (Phillipsburg NJ, USA). HPLC Formic acid was from BDH Industries (Mumbai, India). Analytical grade ammonia solution was from Merck (Darmstadt, Germany). MS formic acid, MS acetic acid, MS ammonium formate, and MS ammonium acetate were from Sigma–Aldrich. Agilent® Technologies (California, USA) was used to perform the chromatography. The machine consists of a vacuum degasser, a binary LC pump, HiP-ALS, 1290 Thermostat, a temperature-controlled autosampler set at 20°C, and a temperature-controlled column compartment set at 40°C. Analyst® Software version 1.6.2 was used to perform data acquisition and quantitation. The compounds were separated on a ZIC®-HILIC column (5 µm, 200 Å 250 × 2.1 mm) protected by a ZIC®-HILIC guard column (14 × 1 mm) (SeQuant, Umea, Sweden) under gradient analysis condition using two mobile phases containing 20 mM ammonium formate added 0.15% formic acid as mobile phase A, and acetonitrile (MS grade) as mobile phase B, at a flow rate of 0.5 mL/min within 10 min. Detection was performed by an AB SCIEX 3200 QTRAP LC/MS/MS system with electrospray ionization source (ESI), multiple reaction monitoring (MRM) scan type, interface operated in the negative ion mode.

Calibration standard: Standard acidic compounds for the preparation of standard solution as L-lactic acid (LA), *p*-hydroxyphenyllactic acid (pHPLA), α-hydroxybutyric acid (aHBA), β-hydroxybutyric acid (bHBA) from Sigma–Aldrich (St. Louis MO, USA). These four candidate acids in this study have been investigated that there were endogenous compounds within human plasma and urine. Water was used for the preparation of calibration curves. Working solutions were prepare by the stock solutions of all four acids diluted in water. The six-point calibration curve was prepared by the combined working solution of all four acids. Blank sample and a zero sample (blank with and without internal standard respectively) were also included in calibration. The limit of detection (LOD) was prepared by dilution of standard solution two (STD2) at 2 and 4 times for higher and lower LOD respectively. LOD is the lowest concentrations that could be able to distinguish clearly from baseline noise. Calibration solutions were prepared, aliquoted, and stored at -80ºC until analysis.

Internal standard: Stable isotope-labeled internal standards (SIL-IS) of the three acids were added in order to compensate the analytes that may lose during sample prepareation procedures. L-lactic-3,3,3-d3 acid (LA-D3) was from Sigma Aldrich, β-hydroxybutyric acid-d4 (bHBA-D4) was from Medical Isotopes (Pelham NH, USA), and [ring-U-$^{13}C_6$]-2-hydroxy-3-(4-hydroxyphenyl) propanoic acid (pHPLA-$^{13}C_6$) was from ALSACHIM (Illkirch-Graffenstaden, France). The SIL-IS stock solutions were prepared

in water. Since the chemical properties of aHBA and bHBA are similar with closely approximate retention time, and same target mass so, these two compounds share the same SIL-IS which is bHBA-D4. All these three internal standards solutions (IS solution) were mixed to prepare combined IS solution then aliquoted, and stored at at -80°C until analysis.

Quality controls: QC solutions were diluted in water and prepared with three different levels as low, middle, and high concentrations of combined working solutions of four candidate acids. There were then aliquoted and stored at –80 °C until analysis.

RESULTS

LA was found to be a statistically significant (*P < 0.05)* parasite product and change increasingly in every stage as displaying in figure 2. pHPLA was shown to be detectable but its concentration was lower than LOD and not significantly different from control group. No statistically significant traces of other acids were found with this specific LC-MS method even when using isolated parasites and higher parasitaemia levels.

Concentrations of each analyte were quantitated by using 6-point calibration curve as previously described above in calibration standard, while

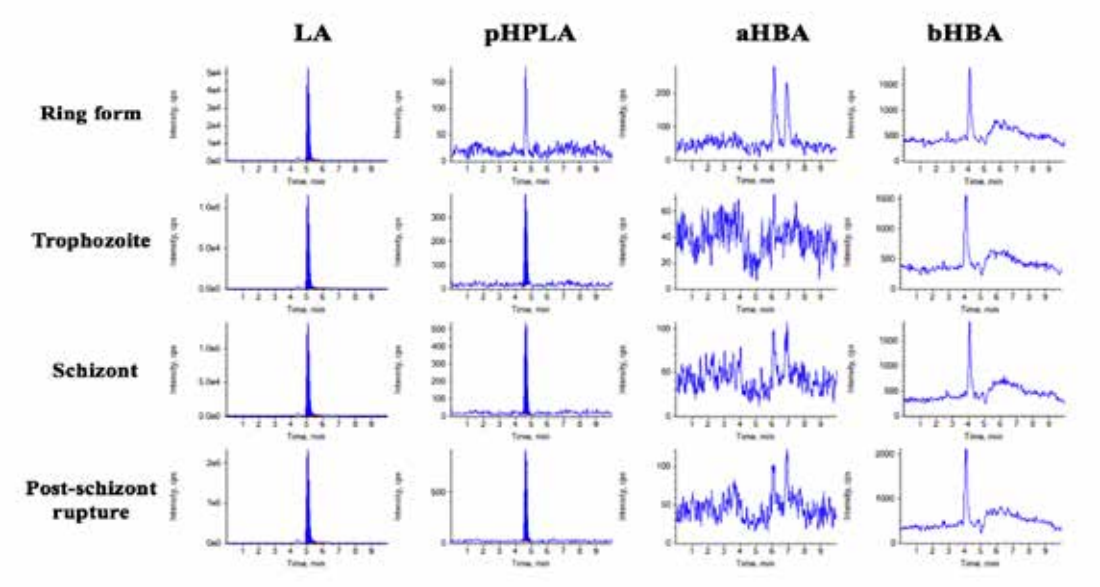


Fig 2- LC-MS peak review of 4 candidate acids shows high intensity and clearly distinguishable peak from back ground noise of LA among each stage.

Table 1 Method specification and calibration range of four organic acids compared with the concentrations found in supernatant from controls and TM267.

Organic acids	Molecular weight (Da)	t_R (min)	LOD	Method calibration range (µg/mL)	Geo. mean concentration in pRBCs (µg/mL)				Geo. mean concentration in Pf. culture (µg/mL)			
					Ring	Troph.	Schiz.	Reinv.	Ring	Troph.	Schiz.	Reinv.
LA	90.1	5.09	10.7	43 – 2,246	119.5	367.5	474	689.5	150.5	642	902.5	1,420
pHPLA	182.2	4.65	0.299	1.20 - 117	No peak	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
aHBA	104.1	3.60	0.283	1.13 - 171	No peak	No peak	No peak	No peak	No peak	No peak	No peak	No peak
bHBA	104.1	2.68	0.808	3.23 - 316	No peak	No peak	No peak	No peak	No peak	No peak	No peak	No peak

t-Test for two-sample assuming equal variances was used as the statistical analysis for comparing the different concentration of sample between control group and TM267 cultivation.

Lactic acid (LA) concentration was found and significantly different between controls and TM267 cultures. There was 150.5 μg/mL of LA measured among TM267 ring stage (119.5 μg/mL in control with $p = 0.02$). While trophozoite and schizont were 642 and 902.5 μg/mL (367.5 and 474 μg/mL in control group with $p = 0.001$ and 0.004) respectively. And 1,420 μg/mL among post-schizont rupture stage (689.5 in control with $p = 0.009$).

DISCUSSION

Only lactic acid (LA) was apparently found in supernatant from Plasmodium falciparum strain TM267 cultivation in accordance with many previous literatures described that LA is a byproduct releasing from parasite through glycolysis pathway after glucose consumption. While other organic acids including p-hydroxyphenyllactic acid(pHPLA), α-hydroxybutyric acid (aHBA), β-hydroxybutyric acid (bHBA) were not significantly discovered by LC-MS among the samples from parasite cultivation. However, there were many possible explanations to support the results before summarizing that these acids are produced or not produced by P. falciparum. First, supernatant was the culture medium which composed of RPMI 1640 basically. One of the RPMI 1640 composition is amino acids such as cystine, glutamate, glutamine, isoleucine, methionine, proline, and tyrosine. This is a significant point to realize that amino acid might compete with the organic acids in supernatant at ion exchange site in SPE cartridge causing un-elution of the 3 candidate acids including pHPLA, aHBA, and bHBA that were not measured at the significant levels. The way for further study is to use culture medium removed amino acids to perform for feeding the parasite, and for the collection of supernatant, but the thing is culture medium without amino acid might not support the parasite growth as good as possible. Second, parasite possibly may not produce those acid by the reason that this study operated TM267 culture with 1% P that was even higher than the parasitamia in the sample from study of M. Trent Herdman and colleague (Herdman MT, *et al.*, 2015) which could measure pHPLA, aHBA, bHBA at concentration 0.000783, 0.00479, and 0.0107 μg/mL respectively among uncomplicated malaria cases with geometric mean of parasitaemia at 11,184 count/μL (approximately 0.2 %), and

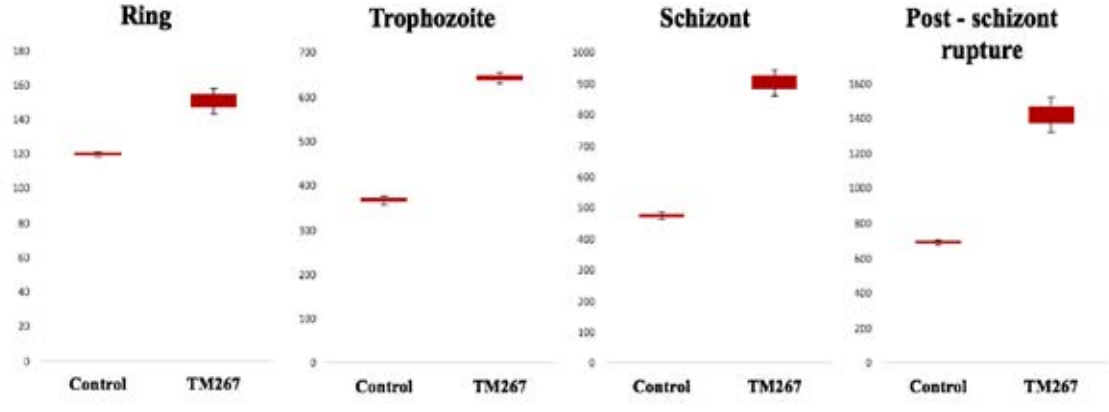


Fig 4- Concentration of lactic acid (LA) comparing between control and TM267 among four stages using *t*-Test for two-sample assuming equal variances.

0.00193, 0.0122, and 0.0145 μg/mL respectively among severe cases with geometric mean of parasitaemia at 27,933 count/μL (approximately 0.5 %). On the other hand, the study of M. Trent Herdman did not measure the acid in supernatant from malaria culture same as this study, the acids that they found were possibly from host response. Lastly, it is about stability. Some acids might be fragile which were probably easy to oxidize with oxygen in the air or other possible reaction. Acid lability and stability need to be further investigated. Lactic acid both L- and D- forms can be detected by LC-MS method. L-lactate can be produce from human metabolic pathways, while both isomers can be found in P. falciparum as the byproduct after glucose consumption to meets its energy supplies (Herdman MT, *et al.,* 2015). However, the effect of D-lactate on the role in pathogenesis of severe malaria still needs further study to confirm. pHPLA was indicates as criteria to severe malaria by its higher levels in plasma and urine, and it was also a diagnostic tool to predict the fatality in patients (Bender DA., 2015). In term of human metabolic pathway, L-tyrosine is a precursor of pHPLA (Herdman MT, *et al.,* 2012) and some metabolic disorder can be the cause of high levels of pHPLA such as tyrosinaemia type III deficiency which is a rare disease found in inborn patients that has a significantly clinical feature in urinary excretion of pHPLA (Endo F, *et al.,* 1983). An enzymatic protein that change hydroxyphenylpyruvate (HPPA) to homogentisate called 4-hydroxyphenylpyruvate dioxygenase. Depletion of this enzyme can be found in patients with neurological impairment causing conversion of HPPA to pHPLA (Lopansri BK, *et al.,* 2006). Some microorganism or normal flora living in gastrointestinal organs such as E. coli, E. faecalis, and lactobacilli can produce pHPLA (Beloborodova N, *et al.,* 2012) so, the morbidity of gastrointestinal vasculature such as sequestration at this particular site can cause the ischaemia leading this normal flora enter the blood stream (Seydel KB, *et al.,* 2006). High levels of pHPLA can be found in plasma of those with this complication consequently. Complicated malaria patients are normally found with high levels of bHBA but its concentration was not significantly associated with fatality (Herdman MT, *et al.,* 2015). The liver is an essential organ to synthesize bHBA using free fatty acid. Whereas aHBA can also be higher in severe cases but its concentration was not involved with mortality rate among the patients as well (Herdman MT, *et al.,* 2015). In term of metabolomics, methionine and threonine are the precursors of aHBA production, and converting of cystathionine to cysteine of glutathione synthesis partway can also produce aHBA (Lord RS, *et al.,* 2008). In addition, urine samples from patients with lactic acidosis have been investigated to be raised levels of aHBA.

CONCLUSION

Lactic acid was evidently detectable as a *P. falciparum* releasing product in accordance with several previous literatures. However, other organic acids including pHPLA, aHBA, and bHBA have not been ensured and concluded that there were not produced by parasite even it was unable to discover by this specific LC-MS method. RPMI 1640 removed amino acids was essential to serve as parasite culture medium before sample collection in order to avoid the competition of amino acids and the candidate analytes at ion exchange site during SPE process. Different strain and species are required to achieve other aspects of acid production from parasite which potentially contributes to metabolic acidosis. The LC-MS method for quantitation was validated of accuracy and precision in order to correctly quantitate the concentration of four organic acids in supernatant from malarial parasite cultivation. This method could be utilized for investigation the metabolic mechanism of acid production within parasite. The method presented high sensitivity of detection, reproducibility, and was appropriate for small volume of sample (100 μL). The method also performed in 96-well plate, which could be high-throughput instrumentation for general bioanalysis study. However, full validation of the method is required to increase reliability and exactness

of quantitation. This specific LC-MS method provides a useful instrument for the detection and quantitation of unknown organic acids in aqueous sample from malaria culture, and will be able to perform further assessment of their metabolism.

ACKNOWLEDGEMENT

This study was supported by Malaria Laboratory, and Clinical Pharmacology laboratories which is granted by Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University.

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