CHAPTER III
EXPERIMENTAL

3.1 Development and Validation of Analytical Method

3.1.1 Instrument and Apparatus

3.1.1.1 Liquid chromatography (LC): Hitachi LaChrom® Elite system consisting of a binary LC pump, a vacuum degasser, an autosampler, and a column oven, Hitachi High Technologies America, Pleasanton CA, USA.

3.1.1.2 Mass spectrometry (MS): Esquire 4000 ion trap mass spectrometer with electrospray ionization source (ESI) interface and QuantAnalysis® version 1.7 software processing, Bruker Daltonics, Bremen, Germany.

3.1.1.3 LC column: ZIC®-HILIC, 250 mm × 2.1 mm, 5 µm, SeQuantTM, Umea, Sweden.

3.1.1.4 LC pre column: ZIC®-HILIC guard column, 20 mm × 2.1 mm, 5 µm, SeQuantTM, Umea, Sweden.

3.1.1.5 Infusion pump: Model 789100C, Harvard Apparatus, Holliston MA, USA

3.1.1.6 Nitrogen gas generator: Model UHLCMS18, Bruker Daltonics, Bremen, Germany.

3.1.1.7 Air compressor for nitrogen gas generator: Model 4000-40M, Bruker Daltonics, Bremen, Germany.

3.1.1.8 Nitrogen gas: ultrahigh purity grade (99.999%), Linde PUB CO., LTD, Bangkok, Thailand.

3.1.1.9 Helium gas: ultrahigh purity grade (99.999%), Linde PUB CO., LTD, Bangkok, Thailand.

3.1.1.10 Multi-stepper pipette: Eppendorf, Hamburg, Germany.

3.1.1.11 Eppendorf MixMateTM: Eppendorf, Hamburg, Germany.

3.1.1.12 ISOLUTE PE-AX 96-well SPE plate: Biotage, Uppsala, Sweden.

3.1.1.13 TurboVap Evaporator: Model TurboVap 96, Caliper, Massachusetts, USA.

3.1.1.14 Vortex mixer: Model G560E, Scientific Industries Inc., New York, USA.

3.1.1.15 Centrifuge: Jouan B4i Multifunction, Thermo Fisher Scientific, New York, USA.

3.1.1.16 Analytical balance B 120L: Sartorious AG, Goettingen, Germany.

3.1.1.17 Analytical balance MC5: Sartorious AG, Goettingen, Germany.

3.1.1.18 pH meter: Mettler Toledo, Bangkok, Thailand.

3.1.1.19 Micropipettes 2-20 µL, 20-200 µL and 100-1,000 µL and tips: Eppendorf, Hamburg, Germany.

3.1.1.20 Polyamide membrane filter 47 mm, 0.45 µm: Sartorious AG, Goettingen, Germany

3.1.1.21 Seal mat: NuncTM, Thermo Fisher Scientific, New York, USA.

3.1.1.22 Silicone seal mat: Altec®, Altec Industries, Inc., Birmingham, USA.

3.1.1.23 Glass 96-well 1 mL: Chrom Tech, Inc., Minnesolta, USA

3.1.1.24 Centrifuge tubes 15 mL: Sterilin®, Thermo Fisher Scientific, New York, USA.

3.1.1.25 Cryogenic vials 2.0 mL: Corning®, Sigma-Aldrich, St. Louis MO, USA.

3.1.1.26 Vial 2.0 mL with screw cap: Agilent Technologies, California, USA.

3.1.1.27 Vial insert 250 µL pulled point glass: Agilent Technologies, California, USA.

3.1.1.28 Beaker 50, 100, 200 and 500 mL

3.1.1.29 Graduated cylinder 100, 500 and 1,000 mL

3.1.1.30 Spatulas

3.1.2 Chemical and Materials

3.1.2.1 Standard Compounds and Stable Isotope-labeled Internal Standard (SIL-IS) Compounds

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) were obtained

from Sigma-Aldrich (St. Louis MO, USA). Stable isotope-labeled internal standards (SIL-IS) were obtained for all eight acids: L-lactic-3,3,3-d3 acid (LA-D3) from Sigma-Aldrich; β-hydroxybutyric acid-d4 (bHBA-D4) and ethyl-d5-malonic acid (EMA-D5) from Medical Isotopes (Pelham NH, USA); malonic acid (MA-13C2), methyl-d3-malonic acid (MMA-D3) and α-ketoglutaric acid disodium salt (1,2,3,4-13C4) from Cambridge Isotope Laboratories, USA); and [ring-U-13C6]-2-hydroxy-3-(4-hydroxyphenyl)propanoic acid (pHPLA-13C6) from ALSACHIM (Illkirch-Graffenstaden, France).

3.1.2.2 Other Chemicals

Acetonitrile (HPLC and MS grade), water (HPLC and MS grade) and methanol (HPLC grade) were obtained from J.T. Baker (Phillipsburg NJ, USA). Formic acid (HPLC grade) was from BDH Industries (Mumbai, India) and ammonia solution (HPLC grade) was from Merck (Darmstadt, Germany). Acetic acid (MS grade) and ammonium acetate (MS grade) were obtained from Sigma-Aldrich.

3.1.3 Preparation of Calibration Standards, Internal Standards and Quality Control

3.1.3.1 Calibration Standards

All eight organic acids in this study were endogenous compounds present in blank plasma and urine from healthy volunteers. Calibration curves were therefore prepared in water, as analyte-free surrogate matrix. Stock solutions of all eight standards were diluted in water to prepare working solutions. Finally, combined working solutions of all eight acids were prepared to build the six-point calibration curve (6 non-zero samples). The ranges were 2.5 to 2,500 µg/mL for LA; 0.125 to 125 µg/mL for aHBA; 7.5 to 375 µg/mL for bHBA; 0.1 to 100 µg/mL for pHPLA; 1 to 1,000 µg/mL for MA; 0.25 to 250 µg/mL for MMA; 0.25 to 100 µg/mL for EMA; and 30 to 1,500 µg/mL for aKGA. The calibration also included a blank sample (blank without internal standard) and a zero sample (blank with internal standard). Calibration solutions were prepared, aliquoted and stored at −80°C until analysis.

3.1.3.2 Internal Standards

The stable isotopically labeled internal standard (SIL-IS) stock solutions were prepared in water. Since aHBA and bHBA presented the same target mass (m/z), close retention times, and similar chemical properties, they shared the same SIL-IS (bHBA-D4). A combined working solution of all 7 SIL-IS; LA-D3 (500 µg/mL), bHBA-D4 (25 µg/mL), pHPLA-13C6 (10 µg/mL), MA-13C2 (100 µg/mL), MMA-D3 (50 µg/mL), EMA-D5 (20 µg/mL), and aKGA-13C4 (300 µg/mL) was prepared, aliquoted and stored at -80°C until analysis.

3.1.3.3 Quality Control

Three quality control (QC) samples containing low, middle and high concentrations of the eight acids were prepared in water at the following concentrations: 7.5, 1,252 and 2,252 µg/mL for LA; 0.375, 62.6 and 113 µg/mL for aHBA; 22.5, 188 and 338 µg/mL for bHBA; 0.3, 50 and 90 µg/mL for pHPLA; 3, 501 and 901 µg/mL for MA; 0.75, 125 and 225 µg/mL for MMA; 0.75, 50.1 and 90.1 µg/mL for EMA; and 90, 751 and 1,351 µg/mL for aKGA. All QC solutions were prepared, aliquoted and stored at -80°C until analysis.

3.1.4 The Optimum Instrumental Analysis Conditions

LC-MS system was supported by Hitachi LaChrom® Elite for LC interfaced with an electrospray ionization ion trap mass spectrometer by Esquire 4000. Data acquisition was performed using DataAnalysis® software.

3.1.4.1 Optimization MS Parameters

Each eight acid standards 10 µg/mL in mobile phase A (acetonitrile:ammonium acetate 100 mM pH 4.7, 80:20, v/v) was infused at flow rate 4 µL/min by infusion pump into ionization interface of ESI in negative mode before to ion trap MS. ESI conditions were optimized such as nebulizer pressure, drying gas flow, drying gas temperature and capillary voltage. In addition, ion trap MS parameters were optimized such as capillary exit offset, skimmers (1 and 2),

octopole, lens (1 and 2) and trap drive, respectively. All ESI-MS conditions were optimized from each individual compound and implemented in the combined method for all eight acid standards.

3.1.4.2 Optimization LC Parameters

The LC condition for separation of eight target acids was developed in HILIC mode. All eight acid compounds were separated on a ZIC®-HILIC (250 mm × 2.1 mm, 5 µm) protected by a ZIC®-HILIC guard column (20 mm × 2.1 mm, 5 µm).

This study was started and optimized with a condition of types, concentration and pH of mobile phase according to previous HILIC method [11]. In addition, wash step condition was added and optimized for reproducibility of subsequent injections. Run time and flow rate were also optimized for separation of eight acids and washing endogenous interferences from plasma and urine samples.
Parameters used in this study were as follows:
Mobile phase A: Acetonitrile (MeCN): Ammonium acetate 100 mM at pH 4.7, (80:20, v/v) was used to separated mixture acid standards.
Mobile phase B: MeCN: Ammonium acetate 25 mM at pH 4.7, (50:50, v/v) was used in a wash step.
Furthermore, mobile phase A was used for reconstitution prior to LC analysis.

3.1.5 Extraction Method Optimization

Sample preparation was developed for simultaneous determination of eight organic acids using solid phase extraction (SPE) technique. ISOLUTE PE-AX 96-well SPE plate was chosen for cleaning up biological samples (plasma and urine) and trapping all anions from small organic acids with high throughput. The sample extracts were injected into LC-MS system for separation and detection, respectively.

The SPE optimization focused on optimizing the content of formic acid in the elution solvent as follow conditions.
Formic acid (%) in methanol: 5, 10, 15 and 20 % (v/v)

3.1.6 Method Validation for Determination of Endogenous Compounds in Biological Samples

The US FDA guidelines for bioanlytical method validation [38] does not contain any direct recommendation for methods quantifying endogenous compounds in biological fluids. The validation was therefore performed according to available FDA guidance criteria [38] with additional experiments for endogenous compounds based on published methods [39-41]. The calibration curve was prepared in water to avoid the potential bias resulting from endogenous compounds occurring naturally at different concentrations in all sources of blank biological fluids. Additional experiments were performed to determine the differences in recoveries between plasma/urine and water (see section 3.1.6.2) [40, 41]. Furthermore, SIL-IS were used to compensate for any variations during sample processing and to compensate for the matrix differences between samples and calibration curves.

3.1.6.1 Selectivity

Analysis of blank plasma and urine samples and spiked blank samples at LLOQ from 6 healthy subjects each were performed for selectivity test.

3.1.6.2 Linearity of Calibration Curves and Sensitivity

Linearity, accuracy and precision were evaluated using calibration curves in water on four separate occasions. All calibration curves were constructed using the analytical responses (chromatographic peak area ratio between the investigated analyte and the SIL-IS) using a linear regression model with $1/x^2$ weighting. Precision and accuracy at the lower and upper limits of quantification (at LLOQ and ULOQ) were evaluated by analyzing three replicates. The carry-over of all eight acids and their 7 SIL-IS were evaluated by injecting blank mobile phase immediately after the injection of a standard with ULOQ concentration. Over-curve dilution was evaluated at three occasions, at a concentration two times greater than the ULOQ and then diluted five times with HPLC water prior analysis.

The analytical responses of all eight acids in plasma/urine and water matrices were assessed to ensure that the calibration curve built in water

could be used to quantify clinical plasma and urine samples. The slope coefficient (α) of 3-point QC curves for all eight acids spiked in plasma/urine from 6 different healthy sources were compared with their respective curves spiked in water solution; recovery factor (RF) = $\alpha_{\text{spiked plasma/urine}}/\alpha_{\text{water}}$. The back-calculated concentrations (C) of QC samples in pooled plasma/urine, with and without RF correction were used to calculate the sum of the absolute values of relative residuals (RR) to evaluate the two methodologies (%RR = 100 x ($C_{\text{spiked plasma/urine}} - C_{\text{nominal}}$)/$C_{\text{nominal}}$)).

3.1.6.3 Recovery and Matrix Effects

The process efficiency, recovery and matrix effects were determined by comparing the area (A) of analytes in 6 individual sources of healthy blank plasma/urine [39]. Due to the potential presence of endogenous concentrations in the blank matrices, the individual areas of the blank samples were subtracted from all sample values. Each parameter was calculated according to the following formulas: process efficiency (%) = 100 x ($A_{\text{spiked}} - A_{\text{blank}}$)/$A_{\text{neat}}$, recovery (%) = 100 x ($A_{\text{spiked}} - A_{\text{blank}}$)/($A_{\text{post-spiked}} - A_{\text{blank}}$) and matrix effect (%) = 100 x [($A_{\text{post-spiked}} - A_{\text{blank}}$)/$A_{\text{neat}}$ - 1]. A qualitative estimation of the matrix effect was also performed through post-column infusion experiments (infusion of all eight acids) with direct injection of extracted blank plasma and urine samples.

3.1.6.4 Precision and Accuracy

Precision and accuracy of the QC samples were evaluated by analyzing five replicates at three different occasions. Intra-assay, inter-assay, and total precisions were calculated at the 3 QC levels using analysis of variance (ANOVA) in GraphPad PRISM® version 5.03 (GraphPad software Inc, California, USA).

3.1.6.5 Stability

The stability of all eight acids in pooled plasma/urine were evaluated at low and high QC levels by three replicates stored under different conditions and durations: three freeze-thaw cycles, ambient temperature and 4 °C for 48 h. Bench-top stability at ambient temperature before SPE, and stability extracted

samples ready to be injected (in the autosampler at 20 C) were evaluated for 4 h and 36 h, respectively. Long term stability in pooled plasma/urine was evaluated for 1 month. Furthermore, stock solution in water stability was also evaluated for 4 months.

3.2 Clinical Applicability

3.2.1 Sample Collection

For plasma samples; whole blood was collected from study participants via a cannula inserted into a peripheral vein, into lithium heparin tubes and placed on ice for immediate transit to the hospital laboratory. Upon arrival, specimens were spun for 7 minutes at 1100 g in a centrifuge refrigerated to 4°C. Urine was collected from participants as a fresh specimen with a mid-stream urine specimen, into a tube with no additives. Plasma and urine were decanted into 2 mL cryovials, and stored in liquid nitrogen or a -80°C freezer. Transportation of all plasma and urine specimens was undertaken in a liquid nitrogen dry shipper or (for periods of under 36 hours) on dry ice. All samples were taken with the fully informed written consent of either the patient or attendant relative, and were part of prospective clinical investigations in malaria which were approved by the Ethical and Scientific Committee of the Centre for Tropical Diseases.

3.2.2 Sample Preparation

One hundred microliter of combined internal standard solution was added to 100 µL of sample (plasma or urine) in a 96-well plate with an Eppendorf (Hamburg, Germany) stream multi-stepper. An additional 800 µL ammonium acetate buffer (pH 8.0; 2.5 mM) was added with a 12-channel pipette, and the 96-well plate was gently mixed (600 rpm) on an Eppendorf MixMate™ (Hamburg, Germany) for about 10 minutes. The 96-well plate was centrifuged at 1100 g for 10 min, and 1 mL was loaded into a conditioned ISOLUTE PE-AX 96-well SPE plate (Biotage, Uppsala, Sweden). All steps in the solid-phase extraction (SPE) procedure were conducted using a 12-channel pipette, as follows. Conditioning; 1 mL methanol was added to

each SPE well, and a vacuum of 3–5 mmHg was applied until all wells were empty (this step was performed two times). The optimized condition of elution solvent (from section 3.1.5) formic acid/methanol (15:85, v/v) was chosen for conditioning/elution solution and added to each SPE well (vacuum of 3–5 mmHg) to remove unwanted SPE impurities. Ammonium acetate buffer (pH 8.0; 2.5 mM) was added to each well, and a vacuum of 3–5 mmHg was applied until all wells were empty. Loading; 1 mL of sample was loaded into the 96-well and a vacuum of 1–1.5 mmHg was applied for 2 min. The vacuum was increased by 0.5 mmHg every 2 min until all samples had passed through the wells. Washing; 1 mL of water followed by 1 mL of methanol was added to each well (vacuum of 3–5 mmHg). Full vacuum was applied for about 10 min, after which the column tips were dried with tissue paper. Elution; a glass 96-well 1 mL collection plate was inserted into the vacuum manifold, and 950 µL conditioning/elution solvent (formic acid/methanol (15:85, v/v)) was added to each well. A vacuum of 0.5–1 mmHg was applied for 2 min and increased by 0.5–1 mmHg every 2 min until all elution solvent had passed through the plate. The eluates were evaporated to dryness in a TurboVap (Caliper, Massachusetts, USA) using nitrogen gas at 40°C until dryness (approximate 2 hours). Reconstitution; 200 µL mobile phase A (100 mM acetonitrile/ammonium acetate (80:20, v/v), pH 4.7) were added using a multistepper auto pipette, and mixed on a MixMate™ at 800 rpm for approximately 10 min. Finally, 5 µL of the reconstituted extracts were injected into the LC-MS system.

3.2.3 Sample Analysis

The acid compounds were separated under isocratic conditions using a mobile phase A at flow rate 0.5 mL/min within 13 minutes. The column was then washed with mobile phase B at a flow rate 0.4 mL/min for 7 minutes. Before each new injection the LC system was re-equilibrated for 1 minute with the starting conditions (total run time 21 minutes).

Detection and quantification were performed by extracting the target mass (m/z) from the total ion chromatogram (TIC), with the following mass to charge ratio (m/z): 89.1 for LA; 92 for LA-D3; 103 for aHBA, bHBA and MA; 107 for bHBA-D4; 105 for MA-$^{13}C_2$; 180.9 for pHPLA; 186.9 for pHPLA-$^{13}C_6$; 117 for MMA; 119.9 for MMA-D3; 131 for EMA; 136 for EMA-D5; 144.9 for aKGA; and 148.9 for aKGA-$^{13}C_4$,and by using QuantAnalysis® version 1.7.

3.3 Statistical Data Analysis

3.3.1 Clinical Samples in The Study

Groups and numbers of plasma and urine samples were summarized in Table 1.

Table 1 Groups and numbers of plasma and urine samples in this study.

Samples	Groups	Number of Sample
Plasma	Severe malaria	141
	Uncomplicated malaria	87
	Healthy	68
Urine	Severe malaria	133
	Uncomplicated malaria	94
	Healthy	61

3.3.2 Univariate Analysis

Kruskal-Wallis non parametric test and multiple comparisons test between pair of sample groups (severe vs. uncomplicated, severe vs. healthy and uncomplicated vs. healthy) were performed using GraphPad PRISM® version 5.03 (GraphPad software Inc, California, USA). All quantitative data (each eight targeted acid concentrations) were expressed as median with interquartile ranges.

3.3.3 Multivariate Analysis

Concentration data of targeted acids from all samples (plasma and urine) were imported as a data matrix input to MATLAB software version 7.0 (MathWorks, Massachusetts, USA) for pre-processing step before pattern recognition process. MATLAB also used for calculation of unsupervised and supervised pattern recognition.

3.3.3.1 Data Pre-processing

Data matrix from three sample groups of 296 plasma samples with 4 acids (296 X 4; row X column) and 288 urine samples with 6 acids (288 X 6) were pre-processing by these following steps. Transforming the elements step and (row and column) scaling step were executed, respectively.

The comparison of the classification feature for three sample groups expressed as PCA score plots in 2 dimension (D) were performed using different pre-process techniques such as n^{th} root (transforming the elements step), mean centring and standardization (scaling step).

3.3.3.2 Unsupervised Pattern Recognition

Principal component analysis (PCA) was used with all pre-processed data matrix of plasma and urine samples. PCA score plots in 2D and/or 3D were performed for finding possible trend in the samples and bi-plots in 2D were performed for exploring the discriminant power between the variables (targeted acids).

3.3.3.3 Supervised Pattern Recognition

Partial least square analysis (PLSDA) and Linear discriminant analysis (LDA) were applied to construct the predicted model for the classification of two sample groups (malaria patient and healthy) and three sample groups (severe malaria, uncomplicated malaria and healthy), respectively.

Performance indices of PLSDA and LDA model were expressed as sensitivity and specificity. % True positive (%TP) is presented for sensitivity and % true negative (%TN) is described for specificity; sensitivity = (TP + FN)/TP X 100 and specificity = (FP + TN)/TN X 100.

Leave-one-out cross validation was a chosen technique to validate all predicted models from PLSDA and LDA in this study.