CHAPTER I
INTRODUCTION

1.1 Background of the Study

Malaria is the most important parasitic disease affected human. It caused an estimated 216 million cases and 655,000 deaths in 2010 [1]. This disease is widespread in tropical and subtropical regions including Africa, Asia and the Americas. In Thailand, Malaria is found on the borders with Cambodia, Laos and Myanmar with low risk [2]. This disease is transmitted via the bite of a blood-feeding female Anopheles mosquito. The symptoms of disease typically include fever and headache, furthermore in severe cases can cause coma or death. Malaria is caused by Plasmodium protozoa, and there are five species of the genus Plasmodium responsible for malaria infections in human; P. falciparum (producing the most severe symptoms and almost cases of death), P. vivax, P. ovale, P. malariae and P. Knowlesi [3].

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Acidosis is a significant prognostic marker and a major cause of death in severe falciparum malaria [4]. It is a condition which there is too much acid in the body fluids (i.e. plasma and urine) and it is defined as arterial blood gas pH < 7.35 and a plasma bicarbonate concentration < 22 mmol/L [5]. A major contributor to metabolic acidosis in severe malaria is lactic acid. Accumulation of lactic acid is caused by an anaerobic glycolysis because of obstructed microcirculatory flow, resulting from the sequestration of parasitized red blood cells [6, 7]. However, lactic acid alone does not account for the total acids in severe malaria patients. There are other unidentified organic acids (which have strong prognostic significance and independent from lactate concentrations) contribute to the strong anion gap [7, 8]. A previous study [7] in adult patients with severe malaria showed a mean plasma strong anion gap of 11.1 mEq/L whereas only 2.9 mEq/L from lactic acid contributing to the acidosis.

To identify other contributing organic acids, a preliminary screening of plasma and urine from healthy volunteers versus severe malaria patients and a comparison of biochemical pathways related to parasite metabolism and severe human febrile illness were performed in this study. Eight small organic acids were identified for further investigation. These 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). Figure 1 showed chemical structure of these eight small organic acids.

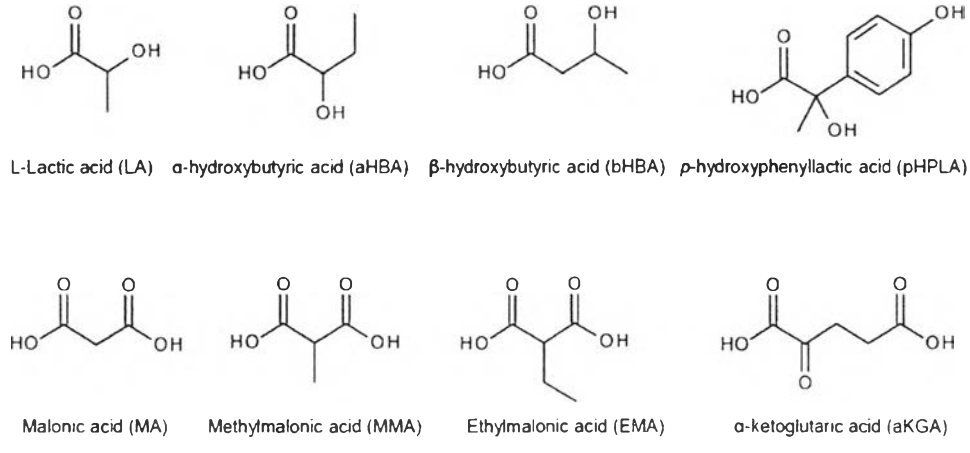

Figure 1 Chemical structures of the eight small organic acids thought to play an important role in the pathophysiology of the metabolic acidosis in severe malaria

The literature is rich of analytical methods developed and validated for the quantification of some of these small organic acids in biological fluids such as liquid chromatography (LC), gas chromatography (GC) and mass spectrometry (MS). However, there are no published methods for the simultaneous quantification of all eight acids. Earlier methods for small polar compounds mostly employed GC-MS [9]. Later, LC-MS based on ion-exchange chromatography methods were developed [10]. The latest methods were LC-MS based on Hydrophilic interaction liquid chromatography (HILIC) [11]. MS is a powerful technique used for many applications because of high sensitivity and selectivity advantages over other techniques.

To obtain the complementary study for an assessment of small organic acids potentially contributor to metabolic acidosis in severe malaria, metabolomics (a powerful approach which combined analytical data with chemometrics for advanced investigations of disease pathophysiology) was performed. Metabolomics is a growing research field focused on the development of analytical methods (LC-MS, NMR, Electrophoresis) to characterise metabolic patterns in various biological systems (i.e. plasma, urine, cerebrospinal fluid). This approach analyzed metabolomic differences between unperturbed versus perturbed systems (such as healthy volunteers vs. patients with a disease) using chemometrics for obtaining reliable results from large amounts of data [12]. Chemometrics is a data analysis approach which applies mathematical, statistical and computational methods to analytical data [13]. Nowadays, one of the most fastest growth, popular and effective technique of chemometrics is pattern recognition [14] thus this technique was used in this study.

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Pattern recognition (unsupervised and supervised) methods have been used for disease diagnosis, prognosis and/or pathogenesis, but there are no published methods for the assessment of potential acidosis contributor in severe malaria. Previous methods have used unsupervised pattern recognition; Hierarchical clustering analysis (HCA) and principal component analysis (PCA) for the assessment of chronic kidney diseases [15]. Supervised pattern recognition; linear discriminant analysis (LDA) has been used for studying the pathophysiology of malignant diseases [16] and partial least square analysis (PLSDA) for the prediction of presence and severity coronary heart disease [17]. In addition, unsupervised and supervised pattern recognition; PCA and PLSDA have been used in the detection of breast cancer [18] and for the identification of biomarker in diabetes [19].

1.2 Literature Reviews

1.2.1 Analytical Method

Previous analytical methods were developed and validated to quantify some of these acids in biological fluids. However, there are no published methods

for the simultaneous quantification of all eight acids hypothesized to play important role in the pathophysiology of the metabolic acidosis in severe malaria.

In 1997, Suh, J.W. et al. [9] presented a screening method for LA, MMA, EMA and other organic acids in urine and cerebrospinal fluid (CSF) using gas chromatography coupled with mass spectrometry (GC-MS). The combination of column and partition chromatography was performed to isolate, purify and quantify these organic acids in urine and CSF. Urine and CSF samples from healthy volunteers were introduced onto cation-exchange column (Dowex 50W X 8 resin) to clean up biological samples before the aqueous eluant was further extracted with ethyl acetate. The organic acids were derivatised into their trimethylsilyl derivatives for GC-MS detection. Recoveries (expressed as mean (%) and %CV) were in the range of 39-70% and 4.3-8.0 %CV.

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In 2000, Magera, M.J. et al. [20] described a new LC-MS/MS method using stable isotope dilution and electrospray tandem mass spectrometry (ESI-MS/MS) for analysis of MMA in plasma. Plasma samples were cleaned up by solid phase extraction (SPE) technique prior injection to the LC-MS/MS system. The separation was achieved on a Supelcosil LC-18, 33 X 4.6 mm column using a 60:40 (v/v) acetonitrile:aqueous formic acid (1g/L) mobile phase. The detection of MMA was performed by selected reaction monitoring (SRM). Mean recoveries of MMA added to plasma were 96.9% (at 0.25 μM) and 94.8% (at 2.02 μM). Inter- and intra-assay were 3.8-8.5 %CV and 1.3-3.4 %CV, respectively.

In 2001, Kushnir, M.M. et al. [21] developed a bioassay of dicarboxylic acids (including MMA) by LC-MS/MS in plasma and urine. Dicarboxylic acids were extracted from biological samples with methyl-tert-butyl ether and derivatized with butanolic hydrochloric acid (HCl) to form dibutyl esters. The derivative was injected into LC-MS/MS for separation and detection. MMA was separated on a Luna C18 column (30 mm x 3.0 mm; 3 μm particles; Phenomenex) with a mobile phase consisting of 850 mL/L methanol and 150 mL/L ammonium formate buffer (0.005 mol/L) at pH 6.5. The ionization and quantification of MMA were performed by TurboIonSprayTM (nebulizer assisted electrospray) ionization and MS/MS in multiple reaction monitoring (MRM) mode. The limit of detection (LOD) and limit of quantification (LOQ) were 0.05 μmol/L and 0.1 μmol/L, respectively. The assay for MMA was linear up to 150 μmol/L.

In 2003, Moreau, N.M. et al. [22] described a GC-MS method for quantification of LA, bHBA (one of the ketone bodies) and short-chain fatty acids in plasma samples. After plasma deproteinisation, a diethyl extraction and a N-tert.-butyldimethylsilyl-N-methyltrifluoroacetamide derivatisation were performed before GC-MS analysis. LOD and LOQ of the assay were 0.7 and 2.5 μM for LA and 49.0 and 73.0 μM for bHBA, respectively. Accuracy, inter- and intra-assay precision were 104.3%, 4.7%CV and 3.3%CV for LA, and 103.7%, 4.3%CV and 3.9%CV for bHBA, respectively.

In 2006, Hilton P.J. et al. [10] presented a study of unexplained acidosis of malnutrition using LC-MS in ion-exchange mode for determination of LA, bHBA and aKGA in ultrafiltrate plasma. Sample analysis was performed on an Aminex HPX-87H Ion exclusion column (300 X 7.8 mm, Bio-Rad) with water titrated to pH 3.2 with concentrated HCl as mobile phase before the eluent was split approximately one-twelfth of the flow to electrospray nebulizer and neutralized by 10 mM ammonium acetate in 50:50 (v/v) methanol-water mixture.

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In 2008, Lakso, H.A. et al. [11] developed a LC-MS method for the quantification of MMA in plasma based on hydrophilic interaction liquid chromatography (HILIC) and ESI-MS. Plasma was deproteinized and the supernatrant injected into LC-MS. Separation was achieved by Merck SeQuant ZIC®-HILIC column with a mobile phase consisting of acetonitrile: 100 mmol/L ammonium acetate buffer (80:20, v/v), pH 4.5 and detection was obtained in negative ESI-MS. Limit of quantification and detection of MMA were 0.09 μmol/L and 0.03 μmol/L, respectively. The linearity of assay was up to 200 μmol/L. Inter- and intra-assay precision at all tested concentration were ≤ 5%CV. Recoveries were in the range of 90-93%.

1.2.2 Pattern Recognition Method

Pattern recognition (unsupervised and supervised) methods have been used for disease diagnosis, prognosis and/or pathogenesis, but there are no published methods for the assessment of potential acidosis contributors in severe malaria.

In 2006, Kirschenlohr, H.L. et al. [17] presented the use of partial least square analysis (PLSDA) with nuclear magnetic resonance (NMR) spectra of blood sera to predict the severity of coronary artery disease (CAD). Determination of the predictive power was performed for defined CAD by using plasma samples from groups of male patients (classified by statin treatment) who had normal coronary arteries (NCA) and CAD. The prediction results for NCA and CAD groups were 80.3% correct for patients not treated and 61.3% for patients treated with statins.

In 2007, Salek, R.M. et al. [19] studied a metabolomic comparison of urinary changed in type 2 diabetes in human, rat and mouse. Principal component analysis (PCA) and PLSDA were used with NMR based metabolomic analysis for the examination of urinary metabolic changes in unmedicated human and two rodent models suffered from type 2 diabetes mellitus and for identification of biomarkers in diabetes.

In 2008, Imre, T., et al. [16] described the use of linear discriminant analysis (LDA) and mass spectrometry for studying the pathophysiology of malignant diseases. The analysis of N-glycan oligosaccharides of human serum α_1-acid glycoprotein samples between three sample groups; healthy individuals, patients with lymphoma and with ovarian tumor were performed. The results of LDA analysis showed a good separation between three groups with 88% classification. The method had predictive power for identifying cancerous vs. healthy individuals showed 96% selectivity and 93% specificity.

In 2011, Gu, H. et al. [18] presented the application of PCA and PLSDA methods for combining NMR and direct analysis in real time (DART)-MS data in in the detection of breast cancer. The metabolomics analysis of serum samples from breast cancer patients and healthy controls was performed. PCA of NMR data showed that the first PC (PC1) scores could be used to separate cancer from healthy samples, however for DART-MS data showed that PC1 could not separate them. Therefore, DART-MS data were reevaluated using orthogonal signal correction from pretreated PLS. This approach using the first latent variable from PLSDA of NMR data caused a significant improvement of the separation between patients and healthy samples, and a metabolic profile related to breast cancer could be extracted from DART-MS.

In 2012, Molin, L. et al. [15] described a comparison between matrix assisted laser desorption ionization (MALDI)-MS and capillary electrophoresis (CE)-MS for biomarker assessment of chronic kidney diseases (CKD). Hierarchical clustering analysis (HCA) and PCA were utilized with (MALDI)-MS data and CE-MS data for detection of biomarkers in urinary proteomics between CKD cases and controls groups. The results showed superior performance of CE-MS in terms of peptide resolution and achievement of disease prediction accuracy rates. However, data from MALDI-MS showed that CKD patients could also separate from controls at slightly less accuracy rates, but expected reduced cost and time.

1.3 Objectives of The Study

Development and validation of analytical method for the qualitative and quantitative assessment of small organic acids in human biological fluids (plasma and urine) by using solid phase extraction (SPE) and liquid chromatography-mass spectrometry (LC-MS) was the main goal of this study. The identification and quantification of small organic acids in plasma and urine samples from patients suffering from metabolic acidosis caused by severe P. falciparum malaria was the following plan. Finally, the use of pattern recognition for finding and identifying patterns of small organic acids in malaria patients versus healthy (control) samples were evaluated.

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1.4 Scope of The Study

This study was started with the finding and hypothesized scope of candidate small organic acids. This was followed by the development and validation of an analytical method which could perform for a simultaneous analysis of small organic acids in human plasma and urine by using SPE for sample cleaning up. LC coupled with MS was used for the separation and detection. The quantification of identified organic acids was accomplished in plasma and urine samples from patients (with severe malaria and also uncomplicated malaria) and healthy samples (control). The study finished with the evaluation of pattern recognition for finding and identifying patterns of organic acids with a significant impact on the metabolic acidosis in

patients with severe malaria. From our findings, interesting pattern recognition could be applied to predict the clinical outcome in patients suffering from P. falciparum malaria.

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