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Liquid chromatographic–mass spectrometric method for simultaneous determination of small organic acids potentially contributing to acidosis in severe malaria☆

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ABSTRACT

Acidosis is an important cause of mortality in severe falciparum malaria. Lactic acid is a major contributor to metabolic acidosis, but accounts for only one-quarter of the strong anion gap. Other unidentified organic acids have an independent strong prognostic significance for a fatal outcome. In this study, a simultaneous bio-analytical method for qualitative and quantitative assessment in plasma and urine of eight small organic acids potentially contributing to acidosis in severe malaria was developed and validated. High-throughput strong anion exchange solid-phase extraction in a 96-well plate format was used for sample preparation. Hydrophilic interaction liquid chromatography (HILIC) coupled to negative mass spectroscopy was utilized for separation and detection. Eight possible small organic acids; 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 analyzed simultaneously using a ZIC-HILIC column with an isocratic elution containing acetonitrile and ammonium acetate buffer. This method was validated according to U.S. Food and Drug Administration guidelines with additional validation procedures for endogenous substances. Accuracy for all eight acids ranged from 93.1% to 104.0%, and the within-day and between-day precisions (i.e. relative standard deviations) were lower than 5.5% at all tested concentrations. The calibration ranges were: 2.5–2500 μg/mL for LA, 0.125–125 μg/mL for aHBA, 7.5–375 μg/mL for bHBA, 0.1–100 μg/mL for pHPLA, 1–1000 μg/mL for MA, 0.25–250 μg/mL for MMA, 0.25–100 μg/mL for EMA, and 30–1500 μg/mL for aKGA. Clinical applicability was demonstrated by analyzing plasma and urine samples from five patients with severe falciparum malaria; five acids had increased concentrations in plasma (range LA = 177–1169 μg/mL, aHBA = 4.70–38.4 μg/mL, bHBA = 7.70–38.0 μg/mL, pHPLA = 0.900–4.30 μg/mL and aKGA = 30.2–32.0) and seven in urine samples (range LA = 11.2–513 μg/mL, aHBA = 1.50–69.5 μg/mL, bHBA = 8.10–111 μg/mL, pHPLA = 4.30–27.7 μg/mL, MMA = 0.300–13.3 μg/mL, EMA = 0.300–48.1 μg/mL and aKGA = 30.4–107 μg/mL). In conclusion, a novel bioanalytical method was developed and validated which allows for simultaneous quantification of eight small organic acids in plasma and urine. This new method may be a useful tool for the assessment of acidosis in patients with severe malaria, and other conditions complicated by acidosis.

1. Introduction

Acidosis is an important prognostic marker in severe falciparum malaria, and a direct cause of death [1]. Lactic acid is a major contributor to metabolic acidosis in severe malaria. Accumulation of lactic acid is caused by anaerobic glycolysis due to obstructed microcirculatory flow, which results from sequestration of parasitized red blood cells [2,3]. However, lactic acid alone does not account for the total acid load in patients with severe malaria, as other yet unidentified organic acids contribute to the

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strong anion gap [2]. The unidentified acids have strong prognostic significance, independent from lactate concentrations [2,4,5]. A previous study [2] in adult patients with severe malaria showed a mean plasma strong anion gap of 11.1 mEq/L, of which only 2.9 mEq/L could be explained by the increased plasma lactic acid concentration.

A preliminary screening of plasma and urine from healthy volunteers and patients with severe malaria, and comparison of biochemical pathways implicated in parasite metabolism and severe human febrile illness, identified eight small organic acids for further investigation in this study. These 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).

Methods have been developed and validated to quantify some of these acids in biological fluids, but there are no published methods for the simultaneous quantification of all eight acids hypothesized to play an important role in acidosis in severe malaria. Previous methods have used gas chromatography–mass spectrometry (GC–MS) [6,7] and liquid chromatography–mass spectrometry (LC–MS) with ion-exchange separation mode [8,9]. Lakso et al. quantified methylmalonic acid in human plasma using hydrophilic interaction liquid chromatography (HILIC) separation and MS detection in single-stage negative electrospray ionization (ESI) mode [10].

Solid-phase extraction (SPE) is commonly preferred to other conventional techniques (e.g. protein precipitation and liquid–liquid extraction) since it provides cleaner extracts suitable for separation coupled to MS detection. Furthermore, the 96-well SPE format facilitates high-throughput processing for future routine analysis work. For separation and detection, HILIC has shown good retention and resolution of small polar acids [11] and the high content of organic solvent in the mobile phase can enhance the efficiency of the electrospray ionization (ESI) and thus the analytical sensitivity. Ion trap mass spectrometry has limited sensitivity for quantification of targeted acids. However, because of the high full-scan sensitivity and its ability to perform MS^n, this was chosen as the most appropriate tool for the qualitative and quantitative screening of the eight small organic acids thought to play a role in severe malaria. Furthermore, this approach can be readily extended for screening clinical samples to explore other potentially relevant acids.

The objective of this study was to develop and validate a novel LC–MS method for simultaneous and accurate qualitative and quantitative assessment of candidate acids in human plasma and urine hypothesized to be important contributors to severe malaria disease. This method could prove an important clinical tool for studying the pathogenesis of acidosis in malaria and other conditions complicated by acidosis.

2. Experimental

2.1. Chemicals and materials

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); [ring-U-$^{13}C_6$]-2-hydroxy-3-(4-hydroxyphenyl)propanoic acid (pHPLA-$^{13}C_6$) from ALSACHIM (Illkirch-Graffenstaden, France); and malonic acid (MA-$^{13}C_2$), methyl-d3-malonic acid (MMA-D3), and α-ketoglutaric acid disodium salt (1,2,3,4-$^{13}C_4$) from Cambridge Isotope Laboratories, USA). Structures of analytes are shown in Fig. 1. 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.

2.2. Instrumentation for liquid chromatography–mass spectrometry

The chromatography was performed on a Hitachi LaChrom® Elite system consisting of a binary LC pump, a vacuum degasser, a temperature-controlled autosampler set at 20 °C, and a temperature-controlled column compartment set at 30 °C (Hitachi High Technologies America, Pleasanton CA, USA). Data acquisition and quantification were performed using QuantAnalysis® version 1.7 (Bruker Daltonics, Bremen, Germany). The compounds were separated on a ZIC®-HILIC column (250 mm × 2.1 mm) protected by a ZIC®-HILIC guard column (5 μm, 20 mm × 2.1 mm) (SeQuant, Umea, Sweden) under isocratic conditions using a mobile phase containing 100 mM acetonitrile/ammonium acetate (80:20, v/v), pH 4.7, at a flow rate of 0.5 mL/min within 13 min. The column was then washed with 25 mM acetonitrile/ammonium acetate (50:50, v/v), pH 4.7, at a flow rate of 0.4 mL/min for 7 min. Before each new injection, the LC system was re-equilibrated for 1 min with the starting conditions (total run time 21 min).

An Esquire 4000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany), with electrospray ionization source (ESI) interface operated in the negative ion mode, was used for detection. The MS conditions were optimized for all eight acid standards by infusing 10 μg/mL standard solutions in mobile phase at 10 μL/min using an infusion pump (Harvard Apparatus, Holliston MA, USA) connected directly to the mass spectrometer. Dry temperature of desolvation was maintained at 365 °C, the capillary voltage was set at 3000 V, the dry gas was set to 8 L/min, and the nebulizer gas was set to 40 psi. Quantification was performed by extracting the target mass (*m*/*z*) from the total ion chromatogram (TIC), with the following target masses (*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-$^{13}C_4$.

2.3. Preparation of calibration standards, internal standards and quality controls

2.3.1. 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. The analytical response differences between plasma/urine and water were evaluated by a recovery assessment (see also Section 2.5). 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 2.5–2500 μg/mL for LA; 0.125–125 μg/mL for aHBA; 7.5–375 μg/mL for bHBA; 0.1–100 μg/mL for pHPLA; 1–1000 μg/mL for MA; 0.25–250 μg/mL for MMA; 0.25–100 μg/mL for EMA; and 30–1500 μg/mL for aKGA. The calibration also included a blank sample (blank without internal standard) and a zero sample (blank with internal standard). The Limit of detection