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.

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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
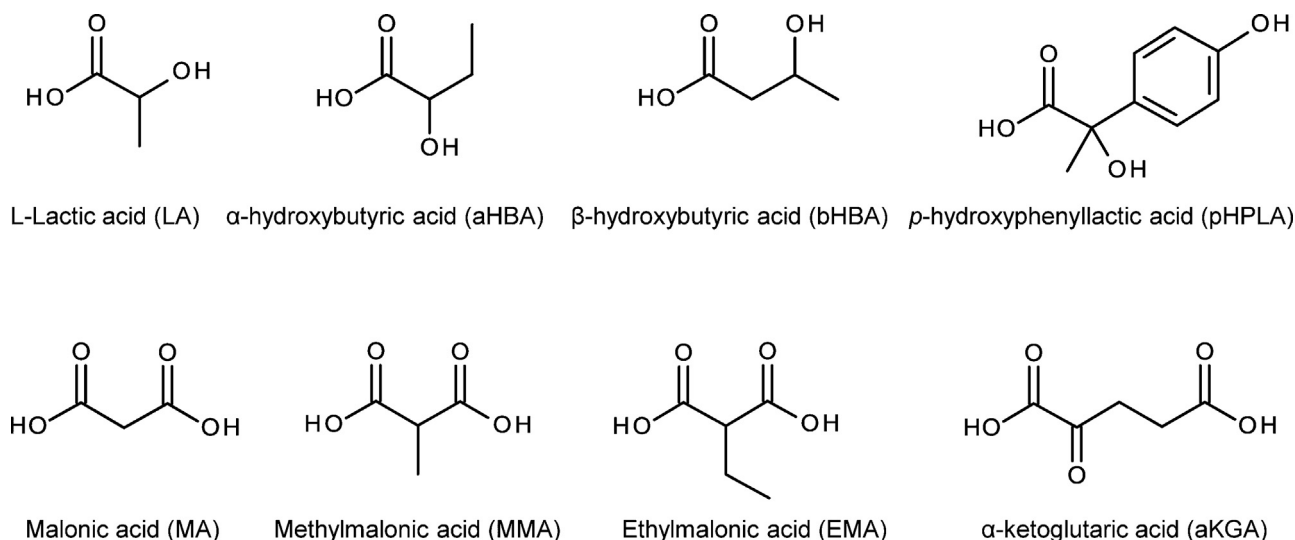
Fig. 1. Chemical structures of the eight potential organic acids thought to play a role in the pathophysiology of the metabolic acidosis in severe malaria.

(LOD) and Lower limit of quantification (LLOQ) were chosen as the lowest concentrations that could be distinguished reliably from the background noise (i.e. signal-to-noise >3 for LOD and >10 for LLOQ) [12]. Calibration solutions were prepared, aliquoted and stored at −80 °C until analysis.

2.3.2. 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-$^{13}C_6$ (10 µg/mL), MA-$^{13}C_2$ (100 µg/mL), MMA-D3 (50 µg/mL), EMA-D5 (20 µg/mL), and aKGA-$^{13}C_4$ (300 µg/mL) was prepared, aliquoted and stored at −80 °C until analysis.

2.3.3. Quality controls (QC)

Three QC samples containing low, middle and high concentrations of the eight acids were prepared in water at the following concentrations: 7.5, 1252 and 2252 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 1351 µg/mL for aKGA. All QC solutions were prepared, aliquoted and stored at −80 °C until analysis.

2.4. 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 min at $1100 \times g$ 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 h) 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 severe malaria which were approved by the Ethical and Scientific Committee of the Centre for Tropical Diseases.

2.5. 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 min. The 96-well plate was centrifuged at $1100 \times g$ 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). Formic acid/methanol (15:85, v/v) conditioning/elution solution was 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 h). Reconstitution; 200 µL mobile phase (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.

2.6. Validation

The US FDA guidelines for bioanlytical method validation [12] does not contain any direct recommendation for the methods quantifying endogenous compounds in biological fluids. The validation

was therefore performed according to available FDA guidance criteria [12] with additional experiments for endogenous compounds based on published methods [13–15]. 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 3.2.2) [14,15]. 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.

Selectivity was evaluated by analysis of blank plasma and urine samples from 6 healthy subjects and also in samples spiked at LLOQ. 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 to evaluate the two methodologies (relative residual = $100 \times (C_{\text{spiked plasma/urine}} - C_{\text{nominal}})/C_{\text{nominal}}$)). 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. 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 (%) = $100 \times (A_{\text{spiked}} - A_{\text{blank}})/A_{\text{neat}}$, recovery (%) = $100 \times (A_{\text{spiked}} - A_{\text{blank}})/(A_{\text{post-spiked}} - A_{\text{blank}})$ and matrix effect (%) = $100 \times [(A_{\text{post-spiked}} - A_{\text{blank}})/A_{\text{neat}} - 1]$. 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.

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).

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 (in the autosampler at 20 °C) were evaluated for 4 h and 36 h, respectively.

3. Results and discussion

The objective of this study was to develop and validate a simultaneous and accurate bioanalytical method for the qualitative and quantitative assessment of eight organic acids in human biological samples (plasma/urine), which might contribute significantly to acidosis in patients with severe malaria.

3.1. Method development

3.1.1. Instrumentation for liquid chromatography–mass spectrometry

The electrospray ionization source (ESI) interface was operated in the negative ion mode to generate, focus and transport the target ions to the ion-trap mass analyzer. This technique offers atmospheric pressure ionization at a low temperature and therefore an efficient ionization of polar compounds. The ESI conditions were optimized (i.e. nebulizer pressure, drying gas flow, drying gas temperature and capillary voltage) for an efficient production of a fine aerosol. The ion trap MS parameters (i.e. octopole, lens and trap drive) were optimized to generate and accumulate the ions to achieve maximum responses of the target ions with the best mass accuracy and resolution. All MS conditions were optimized for each individual compound and implemented in the combined method for all eight acid standards. Even though quantifying eight identified acids, the full mass scan of the ion trap combined with the sample preparation for polar acids allow for identifying other potentially important unknown acids.

All eight organic acids were soluble in water with pKa values ranging from approximately 2.22 to 4.41 [16]. Earlier methods for small polar compounds employed GC–MS. However, GC–techniques require samples to be volatile and have thermal stability, and some samples must be derivatized prior to analysis [6,17,18]. Ion-exchange chromatography requires mobile phases that usually contain high levels of aqueous solvents which could reduce the efficiency of the electrospray ionization (ESI) and thus the analytical sensitivity [8,9].

Because of the polar properties of target analytes, a HILIC method was evaluated. HILIC can efficiently retain and separate small polar compounds, which are not retained in conventional reversed-phase LC [11]. HILIC also requires a high organic content in the mobile phase which potentially increases the desolvation efficiency and enhances the MS sensitivity. In HILIC, the recommended ionic strength for the mobile phases is commonly between 5 and 20 mM but a 100 mM buffer provided a significant improvement in peak shapes (data not shown). The wash step before subsequent sample injection was crucial to avoid endogenous interferences from plasma and urine. This greatly improved the reproducibility of the method and enabled high throughput analysis without degradation of the chromatographic performance or rise in the column back-pressure over an extended period of time.

3.1.2. Sample preparation

The ion-exchange SPE offered cleaner extracts which lead to minimal ion suppression/enhancement for the compounds investigated [19] compared to conventional techniques such as protein precipitation. Smaller volumes are also used with SPE and the technique can be automated using a liquid handler. The SPE optimization focused on minimizing the content of formic acid in the elution since that resulted in the highest and most stable recoveries with appropriate evaporation time (optimal ratio was 15% (v/v) formic acid in methanol).

3.2. Method validation

3.2.1. Selectivity

The chromatograms of all eight acids in blank plasma and urine from healthy volunteers did not show any signals from interference in samples (e.g. concomitant medication), they showed only the endogenous analyte peaks) (i.e. peaks that are found naturally in

Table 1
Method specification and calibration range of eight organic acids compared with the concentrations found in plasma and urine samples from severe malaria patients and healthy subjects (from preliminary analysis).

Acids	Molecular weight (Da)	m/z	t_R (min)	LOD (µg/mL)	LLOQ (µg/mL)	Method calibration range (µg/mL)	Concentration range in plasma		Concentration range in urine	
							Severe malaria patients ($n=5$) (µg/mL)	Healthy subjects ($n=5$) (µg/mL)	Severe malaria patients ($n=5$) (µg/mL)	Healthy subjects ($n=5$) (µg/mL)
LA	90.1	89.1	4.30	0.750	2.50	2.50–2500	177–1169	77.0–113	11.2–513	19.4–30.9
aHBA	104.1	103.0	3.10	0.0375	0.125	0.125–125	4.70–38.4	1.50–3.20	1.50–69.5	2.40–18.8
bHBA	104.1	103.0	3.80	2.25	7.50	7.50–375	7.70–38.0	6.60–9.40	8.10–111	8.10–17.0
pHPLA	182.2	180.9	3.10	0.0300	0.100	0.100–100	0.90–4.30	0.100–0.400	4.30–27.7	0.600–28.2
MA	104.1	103.0	5.70	0.300	1.00	1.00–1000	<LOD	<LOD	<LOD	<LOD
MMA	118.1	117.0	4.60	0.0750	0.250	0.250–250	<LOD	<LOD	0.300–13.3	1.00–5.60
EMA	132.1	131.0	3.00	0.0750	0.250	0.250–100	<LOD	<LOD	0.300–48.1	3.20–16.5
aKGA	146.1	144.9	11.5	9.00	30.0	30.0–1500	30.2–32.0	<LOD	30.4–107	<LOD

LA, L-lactic acid; aHBA, α-hydroxybutyric acid; bHBA, β-hydroxybutyric acid; pHPLA, p-hydroxyphenyllactic acid; MA, malonic acid; MMA, methylmalonic acid; EMA, ethylmalonic acid; aKGA, α-ketoglutaric acid; t_R, retention time; m/z, target mass; LLOQ, lower limit of quantification; LOD, limit of detection.

blank biological samples) within the retention time windows of the chromatographic peaks of analytes and IS. This was also verified during the routine analysis. Average retention times and extract target mass (m/z) of all analytes in six spiked healthy individuals plasma/urine are shown in Table 1.

3.2.2. Linearity of calibration curves and sensitivity

A linear regression model with $1/x^2$ weighting resulted in the best accuracy (93.1–104.0%) and precision (<5.5%) over the entire calibration range and was considered the most appropriate regression model for all eight acids. This was expected considering the heteroscedasticity of data when validating a method over a broad calibration range. The final regression model also resulted in small and evenly distributed residual errors and high coefficient of regression. The accuracy of all eight acids ranged from 92.0–104.0%, 96.0–105.0% and 97.1–104.0% at LLOQ, ULOQ and over-curve, respectively. Inter-assay, intra-assay, and total precision of all eight acids at LLOQ, ULOQ and over-curve were <7.0%, <4.1% and <3.6%, respectively. There were no carry-over effects for any analytes or SIL-IS. The LOD, LLOQ and the calibration ranges of all eight acids are shown in Table 1.

The calibration curves were prepared in water and additional validation procedures were necessary to evaluate the appropriateness of this approach. At QC low, medium and high concentrations, the RF ranged from 0.93 to 1.04 (RSD < 3.9%) in plasma and from 0.97 to 1.01 (RSD < 1.4%) in urine for all eight acids. The implementation of RF for QC samples spiked in plasma/urine did not result in better accuracy or precision; i.e. the absolute sum of relative residuals was higher when implementing a RF than without the correction for both plasma and urine samples (data not shown). Thus, a RF was not necessary despite using different matrices for calibration curves and QC samples compared to clinical samples. Furthermore, adequate accuracy and precision were shown when spiking QC samples in blank plasma and urine and quantifying them using a calibration curve prepared in water (supplementary Table 1). This supports further the parsimonious approach of not compensating for the different matrices.

3.2.3. Recovery and matrix effects

All analytes showed high process efficiency and recovery with no substantial matrix effects in six individual healthy plasma/urine sources: process efficiency = 94.6–99.8% (RSD < 2.7%); recovery = 94.0–99.8% (RSD < 4.1%) and matrix effects = −3.7 to 3.4% (RSD < 4.2%) (supplementary Table 2). The high recovery and lack of matrix effect confirmed that the SPE procedure provided an excellent method for extraction of the target analytes in both urine and plasma samples. Post-column infusions showed that only lactic acid

displayed a clear endogenous peak in the extracted blank plasma but not in urine, due to higher concentrations in blank plasma from healthy volunteers compared to the LOD of the assay (Table 1).

3.2.4. Precision and accuracy

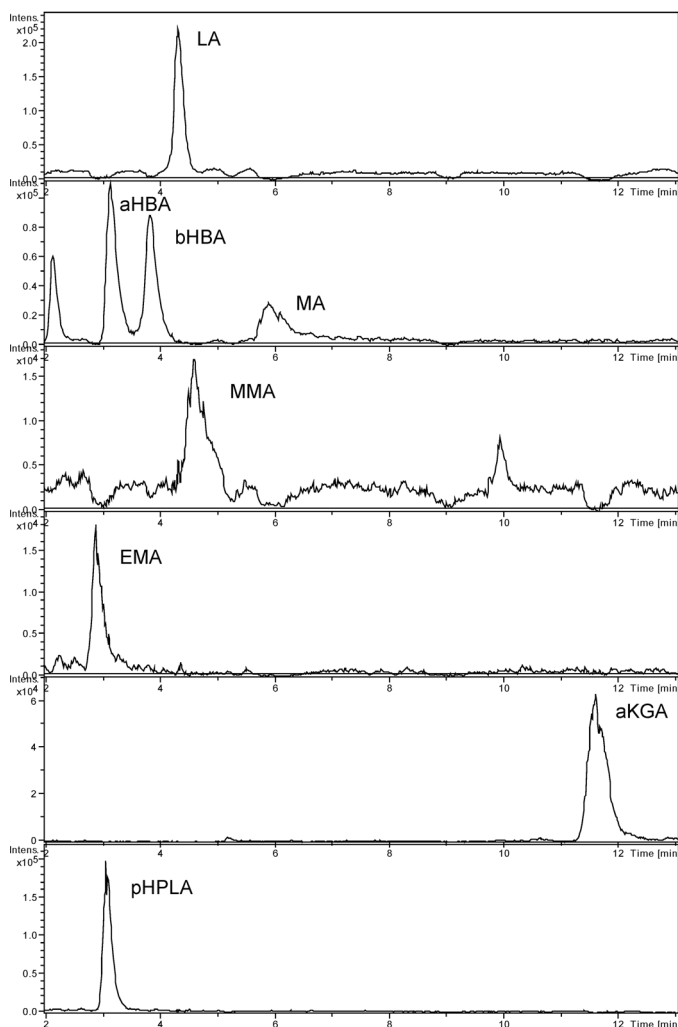The back-calculated concentrations of QC samples prepared in water resulted in acceptable accuracy of 96.4–100.1% with

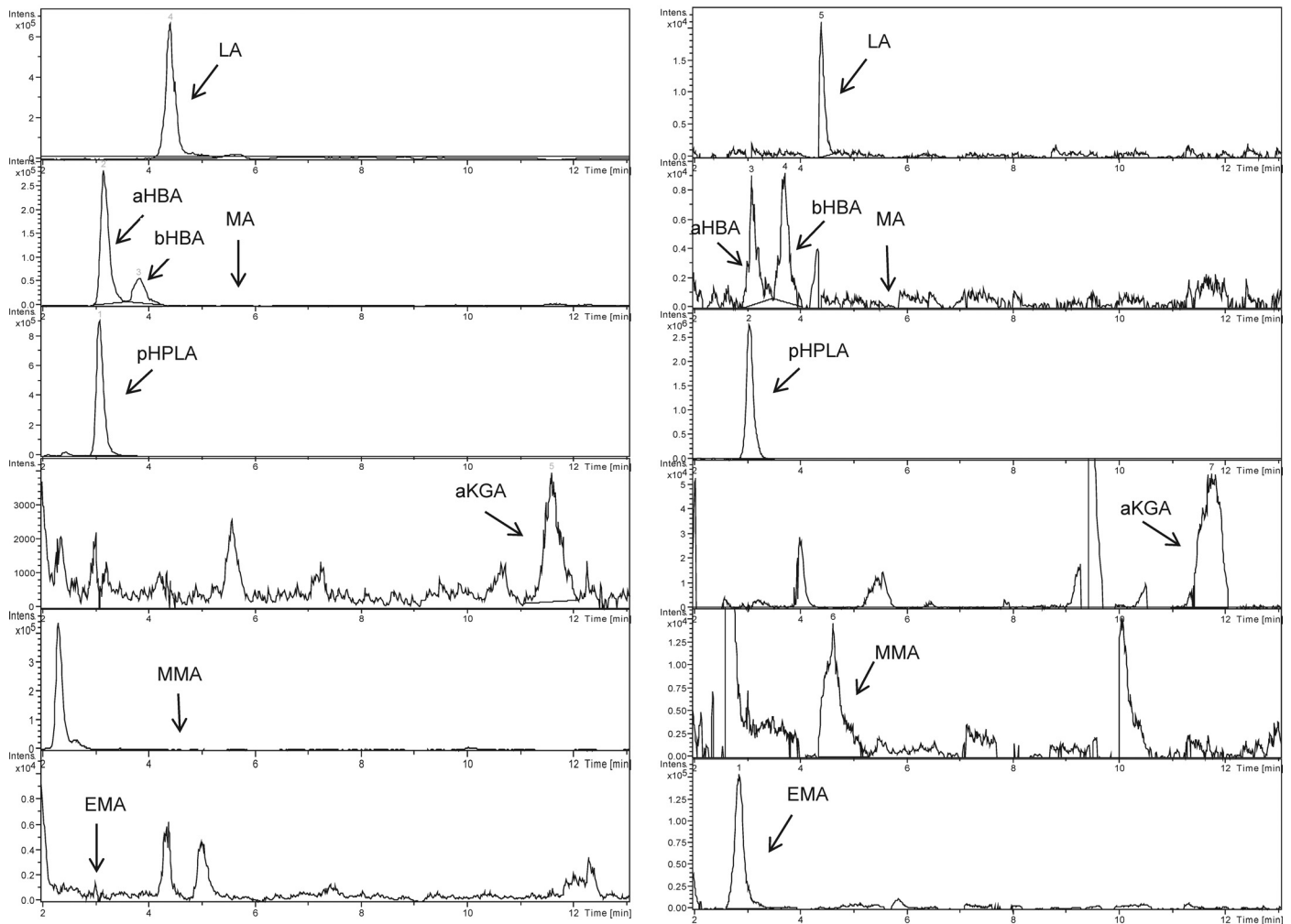**Fig. 2.** Extracted chromatogram of all eight acids spiked in water at QC low level.

Fig. 3. Extracted chromatogram of acids found in a plasma sample (A) and in a urine sample (B) from a patient with severe malaria. Measured concentrations in (A) LA = 1169 μg/mL, aHBA = 38.4 μg/mL, bHBA = 38.0 μg/mL, pHPLA = 4.30 μg/mL and aKGA = 32 μg/mL. Measured concentrations in (B) LA = 513 μg/mL, aHBA = 69.5 μg/mL, bHBA = 111 μg/mL, pHPLA = 27.7 μg/mL, MMA = 13.3 μg/mL, EMA = 48.1 μg/mL and aKGA = 107 μg/mL.

intra-assay, inter-assay and total precisions below 7.6% for all samples (supplementary Table 3). Fig. 2 shows extracted chromatogram of all eight acids spiked in water at QC low level.

3.2.5. Stability

All eight acids remained stable in plasma and urine during three freeze/thaw cycles, at ambient temperature, and also when stored at 4 °C up to 48 h. All acids were also stable at ambient temperature for at least 4 h before SPE, and at 20 °C in the autosampler for at least 72 h. All acids showed long-term stability in plasma and urine matrices for at least 1 month, and in stock solution (water) for at least 4 months. All stability results met FDA acceptance criteria, the differences between the stored and fresh solution of all eight acids in plasma and urine were <8.6% with a precision <7.0%.

3.3. Clinical applicability

The clinical applicability of our method was demonstrated by a preliminary analysis of plasma and urine samples collected in patients with severe malaria from Chittagong, Bangladesh and in healthy volunteers (Table 1). The preliminary results showed that concentration of five of the eight potential acids were significantly elevated in plasma and seven were elevated in urine samples,

respectively. An extracted chromatogram of a plasma and urine sample from a representative patient recruited in the study is shown in Fig. 3. A larger study has been planned to explore and evaluate in more detail the role of these acids in the pathogenesis of severe malaria.

4. Conclusions

An accurate and precise simultaneous LC–MS method was developed and validated for quantitative assessment of eight potential small organic acids in plasma and urine. This method will provide an important tool for studying the pathogenesis of acidosis in patients with severe malaria and other conditions associated with acidosis. The method was shown to be sensitive, reproducible and suitable for small volumes of plasma or urine (i.e. 100 μL). The method utilized SPE in a 96-well format, which permits high-throughput processing and automation of routine analysis of clinical samples. The presented LC–MS method provides an accurate tool for the identification and quantification of as yet unknown acids in patients with severe malaria. This will enable further the assessment of their prognostic and pathophysiological significance in this devastating disease.

Disclosures

None.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.10.005.

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