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Comprehensive quantitative analysis of purines and pyrimidines in the human malaria parasite using ion-pairing ultra-performance liquid chromatography-mass spectrometry

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Abstract

Targeted metabolite profiling has aided in the understanding of a variety of biological processes in the malaria parasite as well as in drug discovery. A fast and sensitive analytical method, based on ion-pairing reversed phase ultra-high performance liquid chromatography tandem mass spectrometry (IP-RP-UPLC-MS/MS), was optimized for the simultaneous analysis of intracellular levels of 35 purine and pyrimidine nucleobases, nucleosides, and nucleotides. This analytical method allows for chromatographic separation of highly polar metabolites using reverse phase chemistry within 15 minutes. The analytical performance of the method was evaluated and successfully applied to the quantification of purines and pyrimidines in *Plasmodium falciparum* and its host cell, the human erythrocyte. In addition, this method can be customized to include other related metabolites such as NADPH and NADP, among others.

Keywords

UPLC-MS; purines and pyrimidines; malaria; *Plasmodium falciparum*; human erythrocytes

1. Introduction

Human malaria is a vector-borne disease caused by five species of parasites of the genus *Plasmodium*. *Plasmodium falciparum* is the most lethal species and accounts for millions of clinical cases and close to a million deaths each year [1]. During the rapid intraerythrocytic asexual stage of malaria infection (blood stages), where the onset of the disease occurs, there is a significant increase in DNA and RNA synthesis, especially during the trophozoite and schizont stages. Therefore, an increased demand for purine and pyrimidine intermediates

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occurs mainly during those stages [2]. *P. falciparum* is a purine auxotroph, salvaging purines from human erythrocytes to sustain DNA and RNA synthesis while pyrimidines are synthesized *de novo* [2]. Liquid chromatography in tandem with mass spectrometry (LC-MS) based approaches to quantify intracellular metabolite levels in the malaria parasite have been used to identify a wide range of molecular classes, including purines, since their biosynthesis has been recognized as a rich source of therapeutic targets for drug development [3–5]; however, a comprehensive purine and pyrimidine quantitative analysis has not been reported.

To date, several methods have been developed for analysis of purines and pyrimidines, including gas chromatography (GC)-MS and LC-MS based methods [6–10]. Purine and pyrimidine nucleobase, nucleoside, and nucleotide quantification have previously been accomplished in cells and foods using ion-pairing chromatography due to the fact that highly charged phosphorylated molecules are retained on a reverse phase column [9–14]. However, the reported methods only account for a small subset of purines and pyrimidines analyzed (up to 24 metabolites), and require long run times, such as 50 minutes [10,11,13,14]. Currently, the simultaneous analysis of tens to hundreds of metabolites is now possible due to continuous technological improvements in both LC resolution, such as ultra-high performance liquid chromatography (UPLC) and high speed mass spectrometers. In addition, modern triple-quadrupole MS can measure positive and negative ions by switching polarities within milliseconds while simultaneously performing full scans for ion product confirmation (PIC) [15]. However, these advances have not yet been fully utilized to develop a comprehensive analytical method for the full spectrum of purines and pyrimidines.

The present study aimed to develop an optimized method for quantification of 35 purine and pyrimidine nucleobases, nucleosides, and nucleotides and be suitable for analysis of a large set of samples. The selected purines and pyrimidines are key metabolites for DNA and RNA synthesis in the malaria parasite [2]. This goal was accomplished using ion pair reversed phase ultra-performance liquid chromatography in tandem with mass spectrometry (IP-RP-UPLC-MS/MS) and using the volatile IP reagent dibutylamine acetate (DBAA). The method was evaluated and applied to the quantification of purines and pyrimidines in *P. falciparum* schizont stage parasites and their host cell, human red blood cells (RBCs). The described method can be applied to many fields, from drug discovery to cell biology, as well as be customized to include other related metabolites such as NADPH and NADP, among others.

2. Materials and Methods

2.1 Materials

All reagents were of the highest commercial quality available. The following reagents were purchased from Sigma Aldrich: nucleobases (adenine, guanine, hypoxanthine), nucleosides (adenosine, thymidine, inosine, uridine, guanosine, cytidine), nucleotides (inosine 5'-monophosphate (IMP), xanthine 5'-monophosphate (XMP), cytidine 5'-monophosphate (CMP), cytidine 5'-diphosphate (CDP), deoxycytidine 5'-diphosphate (dCDP), cytidine 5'-triphosphate (CTP), deoxycytidine 5'-triphosphate (dCTP), uridine 5'-monophosphate (UMP), uridine 5'-diphosphate (UDP), uridine 5'-triphosphate (UTP), guanosine 5'-

monophosphate (GMP), cyclic guanosine 5'-monophosphate (cGMP), guanosine 5'-diphosphate (GDP), deoxyguanosine 5'-diphosphate (dGDP), guanosine 5'-triphosphate (GTP), deoxyguanosine 5'-triphosphate (dGTP), adenosine 5'-monophosphate (AMP), cyclic adenosine 5'-monophosphate (cAMP), adenosine 5'-diphosphate (ADP), deoxyadenosine 5'-diphosphate (dADP), adenosine 5'-triphosphate (ATP), deoxyadenosine 5'-triphosphate (dATP), adenosylsuccinic acid (ASA), thymidine 5'-monophosphate (TMP), thymidine 5'-diphosphate (TDP), thymidine 5'-triphosphate (TTP), [$^{13}\text{C}_9$, $^{15}\text{N}_3$]CTP), and dibutylamine acetate (DBAA). Mass spectroscopy grade acetonitrile, ammonium formate, and formic acid (99%) were purchased from Fisher Scientific. Mass spectrometry grade water was prepared with a Millipore Milli-Q Plus system equipped with an LC-Pak[®] cartridge. O-positive human red blood cells (RBCs) were purchased from The Interstate Companies (Memphis, TN). The following reagents for *P. falciparum* *in vitro* culture were used: Albumax I (Gibco Life Technologies), glucose (Sigma-Aldrich), sodium bicarbonate (Sigma-Aldrich), hypoxanthine (Sigma-Aldrich), HEPES, and gentamicin (Gibco Life Technologies).

2.2 *Plasmodium falciparum* culture conditions and sample collection

Experiments were performed with the *P. falciparum* Dd2 clone as described previously [16]. Briefly, parasites were maintained in O-positive human erythrocytes (4% hematocrit) in RPMI 1640 medium supplemented with 5 g/L Albumax I, 2 g/L glucose, 2.3 g/L sodium bicarbonate, 370 μM hypoxanthine, 25 mM HEPES, and 20 mg/L gentamicin. The parasites were kept at 37 °C under reduced oxygen conditions (5.06% CO_2 , 4.99% O_2 , and 89.95% N_2). Development and multiplication of parasites were monitored by microscopic evaluation of Giemsa-stained thin smears. Ring stage parasites (1–20 h after reinvasion) were synchronized by two treatments with 5% (w/v) D-sorbitol solution in water (5 min at 37 °C) [17].

Schizont forms (30–45 h after reinvasion) were purified using magnetic-activated cell sorting (MACS, Miltenyi Biotec) columns. Briefly, CS columns were placed into the MACS magnetic support and equilibrated with 10 mL of RPMI medium pre-warmed at 37 °C. Parasites from each 20 mL culture (4% hematocrit, 20% parasitemia) were centrifuged at 1000 g for 10 min, resuspended with 5 mL of complete medium at 20% hematocrit, and then loaded on the top of the column. Flow through containing the uninfected RBCs, ring, and young trophozoite infected RBCs was discarded and columns were washed with 20 mL of RPMI medium pre-warmed at 37 °C. Then, 10 mL of RPMI medium pre-warmed at 37 °C was loaded on the top of the column and the column was removed from the magnetic field to elute the schizont forms that were counted using a Neubauer chamber. Parasites were isolated from the host cell by treatment with 0.03% (w/v) saponin for 5 min and pellets were washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.2, at $10,000 \times g$ for 10 min. Samples were kept at – 80 °C until metabolite extraction.

Uninfected RBCs were maintained in complete media at 37 °C in parallel with parasite cultures and recovered by centrifugation at 1000 g for 10 min. Pellets were washed twice with ice-cold PBS, pH 7.2, at $2,000 \times g$ for 10 min and the number of RBCs was determined by counting with the Neubauer chamber.

2.3 Sample preparation

Two separate biological replicates of *P. falciparum* schizont stage parasites (6×10^6 cells) and uninfected RBCs (2×10^7 cells) were extracted. During metabolite extractions, samples were kept on ice and the centrifugation steps were performed at 4 °C as described previously [5]. Briefly, the internal standard [$^{13}\text{C}_9$, $^{15}\text{N}_3$]CTP (CTP-IS) was spiked into each sample for a final concentration of 50 μM after metabolite extraction, which was initiated by adding 0.5 M perchloric acid at 1:7 (v/v, sample/ HClO_4) to the cell pellet, mixed for 10 seconds with a vortex, and incubated on ice for 20 min. Then, extracts were neutralized with 5 M potassium hydroxide at 10:1 (v/v, HClO_4/KOH), mixed immediately for 10 sec, and incubated for an additional 20 min on ice. Samples were then centrifuged for 10 min at 10,000 rpm at 4 °C and supernatants were transferred to an Amicon Ultra (0.5 mL) centrifugal filter and centrifuged for 20 min at 13,000 rpm at 4°C. After filtration, 100 μL of each sample was transferred to a microplate for IP-RP-LC-MS/MS analysis. Injections of 5 μL were performed for both standards and samples. Calibration curves were freshly prepared from stocks and diluted in water.

The stable isotopically labeled nucleotides CTP, UTP, TPP, dCTP, ATP, and GTP were evaluated as internal standards. Any of the mentioned nucleotides can be used as an internal standard using the present method. We selected [$^{13}\text{C}_9$, $^{15}\text{N}_3$]CTP for the present study due to sufficient quantity available in our laboratory at the time of the experiments.

2.4 IP-RP-LC-MS/MS analysis

Separations and analyses were performed using a Waters ACQUITY H-class UPLCTM (Waters, USA) liquid chromatography system in tandem with a XEVO TQ-MSTM mass spectrometer (Waters, USA) equipped with an electrospray ionization (ESI) source. The LC system was equipped with a quaternary pump and autosampler that was maintained at 10 °C. A Waters ACQUITY UPLCTM HSS T3 column (1.8 μm , 2.1 mm $\times$ 100 mm) and an ACQUITY column in-line filter were used. The column temperature was maintained at 40 °C. The standards and samples were separated using a gradient mobile phase consisting of 1.25 mM DBAA, 10 mM ammonium formate in water, and 1% formic acid to adjust the pH to 5.2 (A), and 1.25 mM DBAA, and 10 mM ammonium formate in water:acetonitrile (1:9, v/v) (B). The flow rate was set at 0.3 mL/min and the gradient conditions are summarized in Table 1.

For the MS analysis, the capillary voltage was set at 3.75 kV for positive ion mode and 3.00 kV for negative ion mode. The source and desolvation gas temperatures of the mass spectrometer were set at 150 °C and 450 °C, respectively. The desolvation gas (N_2) was set at 600 L/h. Quantitative determination was performed in ESI positive and negative-ion mode using multiple-reaction monitoring (MRM) mode. The ion transitions, cone voltage, and collision energy used for ESI-MS/MS analysis were determined using MassLynx V4.1 Intellistart software and are presented in Table 2. The use of a quantifier and a qualifier ion per metabolite is recommended for confirmatory purposes but this was not always possible, especially with small molecules with masses below 150 Da. Instead, retention times of the metabolites detected in the samples were compared to the authentic standards to confirm the

analyte identity. In addition, blank samples were run between samples to confirm that the metabolites detected were present only in the samples.

2.5 Data analysis

Data acquisition and analyses were performed using MassLynx V4.1 and TargetLynx software (Waters). Concentration of metabolites was performed by correlating the metabolite:internal standard ratio of MS signals detected by MRM in the calibration curves. The amount of each metabolite detected is expressed as the mean and standard deviation of two biological replicates and two technical replicates run on different days.

2.6 Analytical performance evaluation

We previously reported both metabolite extraction and analysis of purines in uninfected RBCs and *P. falciparum* [5,18]. In addition, the present method was optimized based on the previous report by Yamaoka and colleagues [13]; therefore, only linearity, intra- and inter-day precision, and lower and upper limits of detection/quantification of each metabolite were evaluated. Ion suppression or enhancement caused by matrix interference was evaluated using CTP-IS spiked in uninfected RBCs or *P. falciparum* pellets before extraction and compared to the same amount in water. Intra- and inter- day variation was computed from three independent experiments by the % CV values of the upper limit of quantification from within days and between days using the standard mixture. Limit of detection (LOD) was defined as three times the signal-to-noise ratio and the lower limit of quantification (LLOQ) was defined as 10 times the signal-to-noise ratio [19]. Dynamic range (linearity) and upper limit of quantification was determined by linear regression.

3. Results and Discussion

3.1 IP-RP-LC-MS/MS optimization

We aimed to achieve reduction in sample runtime while effectively resolving 35 purine and pyrimidine nucleobases, nucleosides, and nucleotides. For this purpose, a previous method reported by Yamaoka and colleagues was selected for optimization [13]. Only the mobile phase composition and gradient were optimized while the column type and temperature remained the same. The optimized analytical conditions and retention times for each metabolite are shown in Tables 1 and 2. Previous reports used other ion-pairing reagents such *N,N*-dimethylhexylamine (5–20 mM) [11,20,21] or hexylamine at 5 mM with 0.4% dimethylhexylamine [22]. Similar to dihexylamine acetate used by Yamaoka and colleagues [13], these ion pairs still require long runs to obtain the necessary resolution of nucleotides in a reverse phase column. In a previous report, Klawitter and colleagues used 4 mM dibutylamine formate as the ion-pairing reagent to quantify 11 nucleotides by HPLC-MS/MS [19]. We decided to use dibutylamine acetate (DBAA) to reduce hydrophobic interaction with the stationary phase, therefore, reducing retention times. The acetate salt form of dibutylamine was selected because it has better solubility in water. Based on Yamaoka's report [13], we decided to test DBAA directly at 1.25 mM to avoid contamination on the LC-MS system and this concentration provided excellent resolution of nucleotides in the ACQUITY UPLC™ HSS T3 column. Here, a total runtime of 15 minutes was achieved compared to 50 minutes runtime in the previous method using dihexylamine

acetate (a three-fold decrease in runtime) [13]. Also, an additional 12 compounds could be detected within the same run without decreasing sensitivity [13].

Special attention for chromatographic separation was only needed to resolve ADP from dGDP and ATP from dGTP, as each pair of compounds have the same precursor and product ion (Table 2) [21,23]. Sufficient chromatographic resolution was achieved with the present method to quantify each metabolite (Figure 1), therefore, reducing the risk of inaccuracy due to metabolite cross-talk and in-source fragmentation. We concluded that the selectivity and specificity of the method was satisfactory. Representative chromatograms of the selected 35 purines and pyrimidines for this study are shown in Figure 1.

Reproducibility in retention times among different days was evaluated since the ion pairing approach could be problematic especially due to changes in the concentration of the ion-pairing reagent on the surface of the column material [24,25]. We found that nucleobases and nucleosides presented, on average, less than 0.15 minutes of variability in retention time compared to their phosphorylated counterparts, which varied from 0.5 to 0.9 minutes over the period of 24 hours when only 1.25 mM DBAA was present in eluent B. The addition of 10 mM ammonium formate in eluent B, which was also present at 10 mM in eluent A, reduced the variation in the retention time to less than 0.18 minutes over 48 hours for mono-, di-, and tri-phosphate purines and pyrimidines. The preparation of fresh solvent without the addition of 10 mM ammonium formate to buffer B after 12 hours improved reproducibility and variation was less than 0.1 minutes. However, it required re-equilibration of the LC system reducing the throughput of the method. We conclude that the addition of 10 mM ammonium formate in eluent B significantly improved reproducibility in retention times.

Good peak shape was observed for all purines and pyrimidines with the exception of UMP at concentrations higher than 12.5 μ M (Figure 1). Between 18 and 50 μ M of UMP, the shape of the peak progressively changed from a single peak (0.15 min width) to a broad splitting peak (0.55 min width) (Figure 1). The addition of 10 mM ammonium formate in eluent B or increasing the pH to 6 did not improve the shape of the UMP peak. It is possible that the broad splitting peak is due to the lower strength of retention of DBAA on the ACQUITY UPLC™ HSS T3 column compared to dihexylamine acetate. Because all other metabolites assessed presented good peak shape and resolution and because quantification could be achieved for UMP up to 50 μ M (Figure 2), we did not pursue further optimization.

In addition, our current method offers flexibility since other metabolites with similar chemical properties can also be detected, including methylthioinosine (MTI), methylthioadenosine (MTA), NADPH/NADP⁺, NADH/NAD⁺, as well as methylerythritol phosphate (MEP) intermediates (data not shown). Despite time windows being set for data collection, we found that acceptable dwell times and data points collected for each MRM can be maintained for the simultaneous detection of up to 43 compounds, depending on their retention times, without decreasing sensitivity.

3.2 Analytical performance

The LOD, LLOQ, ULOQ, and linearity for all compounds were evaluated using the optimized IP-RP-LC-MS/MS method. The correlation coefficient (r) for all calibration curves was > 0.98 indicating good correlation between the concentration and metabolite:internal standard ratio of MS signals within the tested ranges (Table 3). In general, the overall LOD and LLOQ increased with the number of phosphates: nucleobases and nucleosides (0.025 – 0.063 μM), monophosphates (0.098 – 1.563 μM), diphosphates (0.781 – 3.125 μM), triphosphates (0.391 – 12.50 μM). The greatest variation was observed among triphosphate intermediates with UTP being the highest LLOQ determined (12.5 μM) (Table 3), similar to previous reports [9,11,13]. A representative calibration curve is shown in Figure 2. Intra-day % CV values ranged between 0.3 and 7.1 % (Table 3). Inter-day variation presented similar values to intra-day variation and ranged between 0.1 and 7.1 %. The % CV values were, in general, below 8%; thus, the method is reproducible and was applied to two different cell types for metabolite quantification.

Because we observed increased LLOQ, mostly for triphosphate compounds, ion suppression or enhancement for triphosphate molecules in the cell matrix was monitored using stable isotopically labeled CTP as the internal standard. [$^{13}\text{C}_9$, $^{15}\text{N}_3$]CTP was spiked into the cell matrix before metabolite extraction and the area of the CTP-IS was integrated in both *P. falciparum* and RBC samples and compared with the CTP-IS area from the same amount spiked in the calibration curves in water. The % CV values were 7.2 for *P. falciparum* and 0.6 for RBC, showing no significant ion suppression or enhancement due to the cellular matrix.

3.3 Purines and pyrimidines levels in *P. falciparum* and uninfected RBCs

The described method was successfully applied to the malaria parasite *P. falciparum* schizont stages and uninfected human RBCs (Table 4). The metabolite levels reported here represent the metabolic state of *P. falciparum* schizont stage and uninfected human RBCs under the culture conditions described in the methods section. We used standard conditions for *in vitro* culture of the malaria parasite where RPMI media was supplemented with 370 μM hypoxanthine, a key precursor for all purine synthesis in *P. falciparum*, and 2 g/L of glucose, which generates ATP through glycolysis [26]. Both metabolites are supplied at non-physiological concentrations; therefore, the *in vitro* metabolic state may differ from the *in vivo* state [5]. Overall, more metabolites were detected and quantified in *P. falciparum* schizont when compared to uninfected human RBCs (Table 4). It was previously shown that many metabolites, including nucleosides and nucleotides, vary during the *P. falciparum* intraerythrocytic cycle with peak abundance during the trophozoite and schizont stages [27]. Under the experimental *in vitro* conditions reported here, the most abundant metabolites in the schizont stage were AMP and ADP (Figure 3 and Table 4). High levels of hypoxanthine were expected since *P. falciparum* salvages purines both from media as well as from the RBC where ATP is in dynamic metabolic exchange with hypoxanthine via ADP, AMP, IMP, inosine, and adenosine [18]. In addition, similar levels of guanine, GMP, GDP, IMP, UMP, and UDP were detected in the parasite (Figure 3). Twelve intermediates were detected below the LLOQ and four intermediates were below the detection limit (Table 4). Our results are consistent with the high demand of RNA and DNA precursors to sustain *P.*

falciparum cell growth and division, in particular adenosine, thymidine, and uridine intermediates, because the parasite contains an (A + T)-rich genome (~80%). Human RBC presented high levels of ATP followed by ADP, AMP, GTP, and IMP (Table 4), similar to previous reports [28]. Nine intermediates were detected below the LLOQ and fourteen intermediates were below the detection limit (Table 4). Human erythrocytes contain millimolar amounts of ATP [28]. During *P. falciparum* infection, erythrocytic ATP is one of the main sources of hypoxanthine, a key metabolite in the purine salvage pathway of the malaria parasite [2].

It is worth mentioning that the metabolite extraction procedure used for infected and uninfected blood can be performed using a 96-well plate format as described previously [5] allowing the simultaneous processing and analysis of hundreds of samples when combined with the present analytical method.

4. Conclusion

The present study aimed to develop an optimized IP-RP-LC-MS/MS method for quantification of 35 purine and pyrimidine nucleobases, nucleosides, and nucleotides and be suitable for analysis of a large set of samples. The method showed versatility and could be customized for other metabolites with similar chemical properties including MTI, MTA, NADPH/NADP⁺, and NADH/NAD⁺, broadening its potential applications. Purine and pyrimidine biosynthesis in the malaria parasite has been recognized as a rich source of therapeutic targets for drug development; therefore, having a robust platform to quantify the parasite's intermediates is of great value. As a proof-of-concept, the method was successfully applied to *P. falciparum* schizont stage parasites and uninfected human RBCs, and it can be expanded to other types of cells and other parasites to monitor response to different metabolic challenges such as purine starvation and drug treatment.

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Abbreviations

IP-RP-UPLC-MS/MS	ion-pairing reverse phase ultra-high performance liquid chromatography in tandem with mass spectrometry
ESI	electrospray ionization
MRM	multiple reaction monitoring
PIC	ion product confirmation
DBAA	dibutylamine acetate
RBCs	red blood cells
CV	coefficients of variation

IMP	inosine 5'-monophosphate
XMP	xanthine 5'-monophosphate
CMP	cytidine 5'-monophosphate
CDP	cytidine 5'-diphosphate
dCDP	deoxycytidine 5'-diphosphate
CTP	cytidine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
UMP	uridine 5'-monophosphate
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate
GMP	guanosine 5'-monophosphate
cGMP	cyclic guanosine 5'-monophosphate
GDP	guanosine 5'-diphosphate
dGDP	deoxyguanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
AMP	adenosine 5'-monophosphate
cAMP	cyclic adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
dADP	deoxyadenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
dATP	deoxyadenosine 5'-triphosphate
ASA	adenosylsuccinic acid
TMP	thymidine 5'-monophosphate
TDP	thymidine 5'-diphosphate
TTP	thymidine 5'-triphosphate

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Highlights of the main findings

- Quantification of 35 purines and pyrimidines accomplished in a 15 minutes runtime
- Versatility to be customized for other metabolites with similar chemical properties
- Broad spectrum of potential applications from drug discovery to cell biology
- The method was validated and applied to malaria parasites and human erythrocytes

Fig. 1.

Combined extracted ion chromatograms of standards of the selected 35 nucleobases, nucleosides, and nucleotides. The corresponding metabolite for each peak is indicated and metabolites were prepared in water at the concentration corresponding to the ULOQ as indicated on table 3. The internal standard [$^{13}\text{C}_9$, $^{15}\text{N}_3$]CTP (CTP-IS) was at 50 μM . The chromatogram for UMP at 50 μM (solid line) and 12.5 μM (dotted line) are shown.

Fig. 2.

Calibration curve for UMP obtained using [$^{13}\text{C}_9$, $^{15}\text{N}_3$]CTP as the internal standard. Response was measured as the ratio between the areas of the analyte and the internal standard.

Fig. 3.

Purine and pyrimidine metabolites quantified in *P. falciparum* schizont stage and RBCs.

Mean values and standard deviations were obtained from two biological replicates and two technical replicates. See Table 4 for values and metabolites that were not detected or quantified.

Table 1

Optimized UPLC inlet method

Time (min) ^a	Percent mobile phase	
	A (%)	B (%)
0	100	0
10	89	11
11	67	33
12	100	0
15	100	0

^aFlow rate was set at 0.3 mL/min

A: Water containing 10 mM ammonium formate and 1.25 mM DBAA (pH 5.2, adjusted with 1 % formic acid)

B: Water:acetonitrile (1:9, v/v) containing 10 mM ammonium formate and 1.25 mM DBAA

Table 2

Analytical conditions and retention times optimized for purine and pyrimidine nucleobases, nucleosides, and nucleotides

Compound	Ion-mode	MRM ^a (m/z)	Cone voltage (V)	Collision energy (eV)	Retention time (min)
Adenine	Positive	135.96 > 118.91	28	18	3.72
Adenosine	Positive	268.10 > 135.97	24	18	6.76
AMP	Negative	346.07 > 78.73	30	20	7.04
ADP	Negative	426.04 > 133.90	34	20	8.70
ATP	Negative	506.00 > 158.79	34	34	10.69
Adenosylsuccinic acid (ASA)	Negative	462.29 > 96.75	32	26	10.29
cAMP	Negative	328.21 > 133.90	36	24	10.72
dADP	Negative	410.20 > 78.79	30	30	9.74
dATP	Negative	490.18 > 158.74	32	24	11.20
Guanine	Positive	152.06 > 79.41	42	26	2.26
Guanosine	Positive	284.10 > 151.97	30	14	4.80
GMP	Negative	362.22 > 78.73	32	20	5.61
GDP	Negative	442.20 > 149.90	34	24	7.67
GTP	Negative	522.18 > 158.79	30	32	9.75
dGDP	Negative	426.20 > 158.74	32	20	8.52
dGTP	Negative	506.18 > 158.73	38	24	10.45
cGMP	Negative	344.20 > 149.89	38	24	8.16
Inosine	Positive	269.23 > 136.95	10	12	4.55
IMP	Negative	347.21 > 78.73	28	24	5.80
Hypoxanthine	Positive	137.11 > 109.93	44	20	2.27
XMP	Negative	363.20 > 210.84	32	20	6.94
Thymidine	Positive	243.23 > 127.00	10	8	6.01
TMP	Negative	321.21 > 78.73	28	16	7.04
TDP	Negative	401.19 > 78.80	36	44	8.66
TTP	Negative	481.17 > 158.73	28	30	10.69
Uridine	Positive	245.20 > 112.96	12	14	2.85
UMP	Negative	323.18 > 96.75	28	18	4.10
UDP	Negative	403.16 > 158.74	32	26	7.41

Compound	Ion-mode	MRM ^a (m/z)	Cone voltage (V)	Collision energy (eV)	Retention time (min)
UTP	Negative	483.14 > 158.74	32	22	9.52
Cytidine	Positive	244.22 > 111.92	28	10	2.14
CMP	Negative	322.20 > 78.78	30	22	2.86
CDP	Negative	402.18 > 158.74	30	28	7.07
CTP	Negative	482.156 > 158.74	34	36	8.91
dCDP	Negative	385.99 > 158.73	32	18	7.32
dCTP	Negative	466.16 > 158.74	36	20	9.21
[¹³ C ₉ , ¹⁵ N ₃]CTP-IS	Negative	493.97 > 158.74	30	32	8.91

^aMRM: multiple reaction monitoring of precursor ion > product ion

Table 3

Parameters evaluated for analytical performance assessment

Compound	LOD (μM)	LLOQ (μM)	ULOQ (μM)	Calibration curve <i>r</i> : correlation coefficient	Intra-day reproducibility % CV of upper limit	Inter-day reproducibility % CV of upper limit
Adenine	0.031	0.031	100	0.999	4.9	2.8
Adenosine	0.025	0.063	50	0.996	4.2	0.3
AMP	0.391	0.781	200	0.989	0.6	1.5
ADP	1.563	3.125	200	0.999	2.4	2.5
ATP	3.125	3.125	200	0.999	0.3	3.2
Adenosylsuccinic acid (ASA)	0.781	1.560	200	0.999	2.8	1.5
cAMP	0.098	0.391	200	0.998	0.8	3.1
dADP	3.125	3.125	200	0.997	0.4	3.8
dATP	3.125	3.125	200	0.995	1.4	3.7
Guanine	0.025	0.063	50	0.996	7.1	5.8
Guanosine	0.025	0.063	50	0.987	0.8	2.5
GMP	0.391	0.781	200	0.995	2.0	3.6
GDP	3.125	3.125	200	0.998	1.3	3.1
GTP	3.125	3.125	200	0.997	3.9	2.4
dGDP	3.125	3.125	200	0.999	1.7	3.0
dGTP	3.125	3.125	200	0.999	1.5	4.1
cGMP	0.098	0.391	200	0.985	1.2	0.8
Inosine	0.031	0.063	50	0.999	1.5	1.3
IMP	0.781	1.563	200	0.996	3.4	2.5
Hypoxanthine	0.025	0.063	100	0.998	2.0	7.1
XMP	0.391	0.781	200	0.998	1.2	1.0
Thymidine	0.250	0.250	50	0.996	1.3	6.2
TMP	0.781	1.563	50	0.987	0.7	2.1
TDP	0.781	1.560	200	0.999	3.1	1.1
TTP	3.125	6.250	200	0.999	3.5	4.0
Uridine	0.031	0.063	100	0.998	1.5	3.3
UMP	0.780	1.563	50	0.999	1.8	1.5
UDP	1.563	3.125	200	0.999	4.2	0.1

Compound	LOD (μM)	LLOQ (μM)	ULOQ (μM)	Calibration curve <i>r</i> : correlation coefficient	Intra-day reproducibility % CV of upper limit	Inter-day reproducibility % CV of upper limit
UTP	3.125	12.50	200	0.999	4.0	6.0
Cytidine	0.025	0.063	50	0.999	3.6	1.4
CMP	0.195	0.391	200	0.999	2.5	1.5
CDP	1.563	3.125	200	0.997	5.2	1.1
CTP	0.391	0.781	200	0.992	1.4	3.7
dCDP	3.125	6.250	200	0.999	5.5	1.0
dCTP	3.125	6.250	200	0.999	3.0	1.3

LOD: Limit of detection; LLOQ: lower limit of quantification; ULOQ: upper limit of quantification

Table 4Purine and pyrimidine levels in *P. falciparum* schizont stage and RBCs

	Mean in <i>P. falciparum</i> (nmol/10 ⁷ cells)	SD	Mean in RBCs (nmol/10 ⁷ cells)	SD
Adenine	0.3	0.1	0.06	0
Adenosine	NQ	NQ	NQ	NQ
AMP	42.7	0.6	1.96	0.21
ADP	24.4	5.1	15.98	0.81
ATP	NQ	NQ	48.58	3.93
ASA	2.2	0.4	0.07	0.01
cAMP	NQ	NQ	NQ	NQ
dADP	NQ	NQ	NQ	NQ
dATP	NQ	NQ	NQ	NQ
Guanine	4.6	3.0	ND	ND
Guanosine	0.9	0.5	ND	ND
GMP	7.5	0.8	NQ	NQ
GDP	5.6	0.8	0.86	0.23
GTP	NQ	NQ	2.00	0.09
dGDP	ND	ND	ND	ND
dGTP	ND	ND	ND	ND
cGMP	NQ	NQ	NQ	NQ
Inosine	1.1	0.4	ND	ND
IMP	7.0	3.5	2.72	0.18
Hypoxanthine	14.8	2.5	8.16	1.61
XMP	NQ	NQ	NQ	NQ
Thymidine	ND	ND	ND	ND
TMP	2.3	0.9	ND	ND
TDP	NQ	NQ	ND	ND
TTP	NQ	NQ	ND	ND
Uridine	1.9	0.9	ND	ND
UMP	7.6	0.7	0.17	0.04
UDP	4.3	1.9	NQ	NQ
UTP	NQ	NQ	0.31	0.13
Cytidine	ND	ND	ND	ND
CMP	1.0	0.1	ND	ND
CDP	1.2	0.3	ND	ND
CTP	0.4	0.1	0.07	0.01
dCDP	1.2	0.4	ND	ND
dCTP	NQ	NQ	NQ	NQ

Mean values and standard deviations (SD) were obtained from two biological replicates and two technical replicates; NQ: detected below the LLOQ; ND: not detected (below LOD)