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Extraction of Hydrophilic Metabolites from *Plasmodium falciparum*-Infected Erythrocytes for Metabolomic Analysis

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Abstract

Metabolomics is an increasingly common analytical approach for investigating metabolic networks of pathogenic organisms. This may be of particular use in the study of parasitic infections due to the intrinsic metabolic connection between the parasite and its host. In vitro cultures of the malaria parasite *Plasmodium falciparum* present a valuable platform to elucidate the structure and dynamics of the parasite's metabolic network and to determine the mechanisms of action of antimalarial drugs and drug resistance mutations. Accurately measuring metabolite levels requires a reproducible method for quantifying intracellular metabolites. Here we present a simple protocol for extracting hydrophilic metabolites from *P. falciparum*-infected erythrocyte cultures.

Keywords

Malaria; *Plasmodium falciparum*; Erythrocyte; Red blood cell; Metabolomics; Mass spectrometry; Liquid chromatography; LC-MS; Nuclear magnetic resonance; NMR

1. Introduction

Metabolomics is an analytical approach used to probe the metabolic networks of a wide range of model organisms, cell culture tissues, pathogens, and clinical patients. Metabolomics has been used for biomarker identification, diagnosis, disease classification, basic biochemical analysis of metabolic flux and regulatory mechanisms, and understanding the mechanisms of drug action and toxicity (1–5). One field where metabolomics is well suited is in the study of parasitic diseases. Parasites are metabolically intertwined with their host, and many antiparasitic pharmaceuticals are anti-metabolites that kill the invading organism by inhibiting parasite-specific metabolic processes. Malaria, which is caused by protozoan parasites of the genus *Plasmodium*, afflicts more than 200 million patients and causes roughly 1 million deaths per year (6). The ongoing crisis of antimalarial drug resistance (7, 8) demands a deeper understanding of the biochemical pathways underlying parasite growth in order to identify novel drug targets.

Systems-level metabolomic modeling has been made possible by recent advances in nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS). Several groups have now used these tools to address questions pertaining to the basic biochemistry of the malaria parasite (9–11), reviewed in ref. 12. We recently reported the first metabolomic analysis of *Plasmodium falciparum* parasites in culture (10). Using an LC-MS analytical platform, we measured the relative levels of roughly 80 metabolites, in both cell extracts and in the culture medium, over the course of the 48-h intraerythrocytic developmental cycle. We observed many metabolic alterations to the host cell, some of

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which were readily explicable in the context of known parasite biology. For example, nucleosides, nucleotides, and their biosynthetic intermediates peaked in abundance at the trophozoite stage, coincident with the process of genome replication. We also observed a novel metabolic phenotype: *Plasmodium* parasites rapidly and specifically degrade extracellular arginine into the non-proteogenic amino acid ornithine, a reaction we determined to be catalyzed by the *P. falciparum* arginase enzyme. This discovery may tie in to the observed correlation between clinical hypoarginemia and malaria infection (13). Further experiments in our lab are focused on using metabolomics to elucidate the influence of genetic variation on metabolic phenotypes, the mechanism of action for poorly characterized antimalarial drugs such as artemisinin and the metabolic consequences of drug-resistance mutations.

All metabolomic analyses depend on reproducible methods for purifying metabolites from complex biological extracts in order to generate high-quality data. To this end, we have adapted an established methanolic extraction protocol for isolating hydrophilic molecules from *Plasmodium*-infected erythrocytes (14).

1.1. Methanolic Extraction of Hydrophilic Metabolites

In this method, parasite-infected erythrocytes are rapidly harvested and extracted in cold methanol at dry-ice temperatures (−70°C), which serves to quench enzymatic activity by quickly dropping the temperature to levels that freeze the cytoplasm. The quenched cells are incubated on dry ice to allow time for lysis to proceed from the freezing- and solvent-induced membrane disruption and the mixing of the cell contents and methanol. A second extraction with sonication is then performed to disrupt cell structures and recover remaining metabolites. Similar cold methanol-based extractions are commonly and successfully used in metabolomic analyses of microbes such as bacteria and yeast (15, 16). We have investigated the use of other extraction solvent mixes such as 50:50 methanol–water, 100% acetonitrile, and 40:40:20 methanol–acetonitrile–water, but found that they are less effective at deproteinating the extract, typically resulting in a pink to reddish solution due to residual hemoglobin. The methanol-based method described here removes protein effectively enough that it does not interfere with our chromatography or analysis. Using this approach, we have quantified relative metabolite levels (10), traced the flow of isotope-labeled substrates through metabolic pathways (9) and detect several thousand distinct mass signatures in infected erythrocytes (unpublished results).

Several limitations of this methodology are worth considering. One biological consideration of our extraction method is that metabolite pools in the host cell cytosol and parasite compartments are mixed during the metabolite extraction. To specifically profile parasite-specific metabolites, it is necessary to isolate the parasite from the host cell material. One example of parasite purification is saponin lysis (e.g., ref. (11)). The obvious disadvantage of saponin lysis is that it subjects the parasite to non-physiological conditions for relatively long periods of time, which may result in alterations of metabolic pools, fluxes or labeling patterns. However, saponin lysis and other related techniques provide a powerful mechanism for differentiating between host and parasite metabolism and quantifying the flow of metabolites between various sub-cellular compartments. Another biological consideration of our sample preparation protocol is that we do not wash our cell pellet prior to lysis and extraction. This is motivated by the desire to quickly quench metabolism in order to capture the state of normal cell metabolism as accurately as possible, without subjecting the cells to non-physiological stresses associated with washing such as nutrient deprivation. Consequently, significant amounts of culture medium are present in the metabolite extracts. This negatively impacts the quantitation of all compounds in cell extracts due to matrix effects, ion suppression, and chromatographic interference by the high-abundance medium

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nutrients. Moreover, it disallows the precise determination of intracellular concentrations of any nutrients present in the medium, principally glucose, amino acids, and vitamins.

A final biological consideration is that in vitro culturing of *Plasmodium* typically produces parasitemia ranging from 1 to 10%. Consequently, the erythrocyte background contributes a significant fraction of the signal observed in metabolomics analysis for certain metabolites. Although various methods are available for producing high-parasitemia cultures, such as magnetic separation (17) and Percoll purification (18), these methods are most effective for late stage parasites.

1.2. Mass Spectrometry and Nuclear Magnetic Resonance Spectrometry

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the most common analytical tools used for metabolomics. Each tool presents certain advantages and disadvantages (19). Briefly, NMR is sensitive to a broad range of molecules, can solve molecular structures de novo, and is quantitatively robust. However, NMR is limited by its low sensitivity, which restricts NMR-based studies to the 30–50 most abundant compounds present in biological extracts (20). In contrast, MS is less quantitatively robust, often cannot uniquely identify a particular compound, and the signal is affected by the inherent inonizability of metabolites. However, even low-end mass spectrometers are several orders of magnitude more sensitive than NMR. Consequently, MS and NMR offer distinct and complementary analytical platforms from which to investigate parasite metabolism.

Both MS and NMR-based approaches have been applied to malaria research. NMR has been successfully employed in analyzing *Plasmodium* metabolism, both in in vitro cultures and in rodent host models (11, 21, 22). Mass spectrometry has also been employed in the study of the parasite and malarial metabolism (9, 10, 23–25). However, global or untargeted mass spectrometric analyses often pose intractable difficulties in the identification of previously uncharacterized metabolites (26). In addition, molar concentrations generally cannot be directly determined from an MS chromatogram, but must be calculated with separate quantification experiments employing spiked-in standards or standard curves on a compound-by-compound basis. Various factors (chromatographic drift, ion suppression, matrix effects, etc.) can also change relative signal over time and negatively impact reproducibility.

Chromatography is another factor that plays a major role in determining which types of metabolites will be observed in an analysis (reviewed in ref. (19)). One significant division is between gas chromatography (GC-MS) and liquid chromatography (LC-MS). GC-MS is capable of profiling most classes of cellular metabolites with a high resolving power, including hydrophobic and lipidic species. However, GC generally requires chemical derivatization to facilitate gas-phase ionization. Furthermore, GC is generally limited to electron-impact ionization, which makes untargeted analysis of complex mixtures impractical due to its tendency to fragment the molecular ion of a species into a number of low-abundance daughter ions. LC-MS, the favored technique in our laboratory, profiles different classes of metabolites depending on the specific column chemistry without the requirement of derivatization (although derivatization may still be useful for specific experiments). Most hydrophilic intermediates in central carbon metabolism can be profiled directly using the extraction protocol outlined here and a combination of reverse-phase and hydrophilic interaction liquid chromatography (14, 27, 28). Furthermore, this protocol can be used for both targeted (e.g., using triple-quadrupole MS, (10)) and untargeted approaches (e.g., using an orbitrap MS, (29)). The specific combination of chromatographic and analytical platform should ultimately be determined by the chemistry of the metabolites of interest.

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2. Materials

Methanol and water used to make the extraction solvent must be HPLC-grade.

1. 100% Methanol. Store at room temperature (RT).
2. 80% Methanol–20% water. Store at 4°C.
3. Dry ice.
4. A water-bath sonicator (see Note 1).
5. Floating Eppendorf tube rack.
6. A nitrogen-flow sample concentrator/evaporator (if drying samples) (see Note 2).

3. Methods

Carry out all procedures at RT unless otherwise specified.

3.1. Preparation

1. This protocol assumes in vitro cultures of *P. falciparum* are grown using standard methods. Each culture harvested should contain at least 50 μl of packed cell volume.
2. For each sample to be harvested, label three 1.5-ml Eppendorf tubes as Tube 1, Tube 2, and Tube 3. If you intend to harvest culture medium for analysis, also label a Tube M.
3. Pipet 450 μl of 100% methanol into Tube 1 and cool on dry ice for at least 5 min. If also harvesting medium, pipet 200 μl of 100% methanol into Tube M (see Note 3).
4. Fill sonicator with water and add crushed ice to cover the water surface.

3.2. First Extraction

1. Remove the culture flasks or plates from the incubator and resuspend cells by gently shaking. Pipet desired volume to a centrifuge tube (see Notes 4 and [5]).
2. Centrifuge tube for 5 min (500 × *g*, RT, low brake).
3. If harvesting culture medium, pipet 50 μl of the supernatant to Tube M, vortex briefly and store on dry ice.
4. Aspirate the supernatant, removing as much as possible without disturbing the cell pellet.

[1] Acceptable sonicators are generally sold as equipment-cleaning devices and have only one frequency. Try to use a sonicator with an operating frequency of at least 40 kHz, such as a Bransonic 3150.

[2] Drying the sample is optional, but may be carried out if the liquid chromatography platform you are using performs poorly with high levels of organic solvent (see Note 11) or if the sample needs to be concentrated (see Note 3).

[3] The volumes of solvent given here are based on the assumption that you will be drying and resuspending your sample, and so if you are not they may give too dilute a sample. If you prefer to run your sample directly, without drying, or if you are harvesting a packed cell volume other than 50 μl, you may use anywhere from 4 to 10 times your packed cell volume of 100% methanol in Subheading 3.1, step 3, and 4–10 times your packed cell volume of 80% methanol in Subheading 3.3, step 1.

[4] This is an excellent time to take a diagnostic smear for a microscope slide.

[5] In general, we find that running at least three biological replicates is desirable to collect statistically meaningful data. We find that up to 12 cultures can be comfortably harvested and extracted at a time.

5. Carefully collect 50 μl of the cell pellet with pipette (see Note 6). Pipet the cells into Tube 1 (see Notes 7 and [8]). Immediately vortex vigorously and return to dry ice.

6. After harvesting all cultures, vigorously vortex Tube 1 for 10 s and return to dry ice. Incubate on dry ice for 15 min, vortexing every 5 min as above.

7. Centrifuge Tube 1 for 5 min (500 × *g*, 4°C).

8. Collect the supernatant with pipette and transfer to Tube 2. Store Tube 2 on dry ice.

3.3. Second Extraction

1. Add 450 μl of 80% MeOH (4°C) to the pellet in Tube 1. Break up the pellet by scraping with the pipette tip, pipetting up and down several times and vortexing.

2. Sonicate Tube 1 on ice for 15 min (see Note 9).

3. Centrifuge Tube 1 for 5 min (16,000 × *g*, 4°C).

4. Pool the extracts by adding the supernatant to Tube 2. Clarify any remaining protein and debris by centrifuging Tube 2 for 10 min (16,000 × *g*, 4°C). If you harvested medium, centrifuge Tube M at the same time.

5. Transfer supernatant to Tube 3. Store at −80°C until ready for analysis. If you harvested medium, transfer the Tube M supernatant to a fresh tube and store as well.

3.4. Sample Drying and Resuspension (Optional) (See Note 2)

1. Transfer Tube 3 to the sample concentrator and dry under nitrogen flow. Adjust the probe height and flow rate so that the flow gently agitates the surface of the liquid (see Note 10).

2. Store dried extract at −80°C.

3. When ready to analyze samples, add 200 μl of HPLC-grade solvent (4°C) to Tube 3 and vortex vigorously until the pellet is completely dissolved (see Note 11).

4. Clarify the sample of anything that failed to resuspend by centrifuging Tube 3 for 5 min (16,000 × *g*, 4°C). The supernatant can then be analyzed on the mass spectrometer of choice or transferred to a fresh tube and stored at −80°C.

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[6] Slowly stirring with the pipette tip will help with pipetting, as the pellet will be viscous. Be very careful to collect consistent volumes across different samples.

[7] Do not submerge the pipette tip directly into the methanol, as this will freeze the sample in the tip and clog it. Instead, hold the pipette tip above the methanol and expel the sample so that it "drips" into the methanol. Afterwards, wash the pipette tip several times by pipetting up and down.

[8] Eppendorf tubes will often become brittle and difficult to open at dry ice temperatures. You may need to warm the lid a few seconds with your fingertips before opening. Alternatively, you may open the tubes prior to harvesting the sample and leave them on the dry ice. This requires special care, however, so that you do not spill the methanol.

[9] Sonication generates heat, so periodically check to ensure that the ice has not entirely melted. Add more ice if necessary.

[10] This process generally takes 45–60 min. You may speed it up by periodically lowering the probe as the solvent level drops.

[11] The specific amount of solvent to add at this step depends on the amount of packed cells that you harvested (see Note 3), the desired degree of concentration and the solvent used and may have to be determined empirically. We typically resuspend in water when conducting reverse-phase HPLC.

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