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Short technical report

A modified precise analytical method for anti-malarial screening: Heme polymerization assay

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ABSTRACT

Malarial parasite detoxifies the heme generated in its food vacuole in many ways one of which involves heme polymerization to hemozoin. The existing heme polymerization assays involve use of activators along with buffers for polymerization of heme leading to its precipitation. Such assays then involve special instruments and laborious work of isolating the precipitated polymer and its detection. Simple and precise spectrophotometric and HTS methods were developed for heme polymerization using tween 20 as the activator without isolation of polymerized heme.

Haemoglobin present in erythrocytes is digested by malarial parasites (*Plasmodium* species) as a source of essential nutrients during development in human body. The parasite degrades haemoglobin to free heme moieties. The heme moieties are toxic in nature because of the ability to produce free radicals as well as reactive oxygen species, which can cause oxidative stress. Numerous detoxification mechanisms have been employed by the parasites like catabolism by heme oxygenase and heme protein binding, biocrystallization to hemozoin, peroxidative degradation and containment in peritrophic matrix. One of the major mechanisms of detoxification involves conversion of free heme to hemozoin (polymerization) which is chemically similar to β-hematin. Hence, blocking formation of β—hematin (hemozoin) is an ideal target for antimalarial screening [1–4].

Heme polymerisation assay is one of the non-cell based mechanistic assay which involves replication of condition of parasite food vacuole by using buffers of similar pH, temperature and activator for polymerization of heme [5–6]. Several other factors such as time of assay, type of activator and concentration of heme are taken into consideration. Activators such as lipids and surfactants have been reported for formation of beta hematin from heme [7–10]. Formation of hemozoin has been mediated by lipids like stearic acid, palmitic acid, oleic acid and linoleic acid. The free form and methyl esters of some of these fatty acids are reported to be present in malarial parasites which are reported to affect heme detoxification. The phospholipids and glycolipids present in malarial parasites have shown a role in hemozoin formation. Several methods for heme polymerization or crystallization have been reported which required isolation of dimeric heme and are therefore laborious with requirement of specialized instruments [11–15]. Some of the spectrophotometric methods involving acetate buffers develop turbidity over the time of assay and so a correction at 630 nm is essential which was not found to be reproducible.

We developed a simple, precise, rapid and reproducible method for heme polymerization. Monomeric heme gets absorbed at 405 nm (Soret band) whereas at 630 nm, polymerized heme gets detected. Hence, heme polymerization was monitored at 405 nm with decrease in absorbance and at 630 nm with increase in absorbance. Initial method development was carried out using spectrophotometric technique and suitable changes were done for high throughput screening method.

Hemin chloride (heme), chloroquine diphosphate (CQ) and quinine sulphate were purchased from Sigma Chemicals. Primaquine phosphate and artemether were commercially obtained. Dimethyl sulphoxide (DMSO) was purchased from Merck. Tween 20 was procured from Himedia. The phosphate buffer solutions of pH 5 and 5.5 were prepared using standard protocols. All chemicals used in analysis were of highest purity and quality. Flat bottom 96 well plastic plates were obtained from Tarsons. Multi well plate reader

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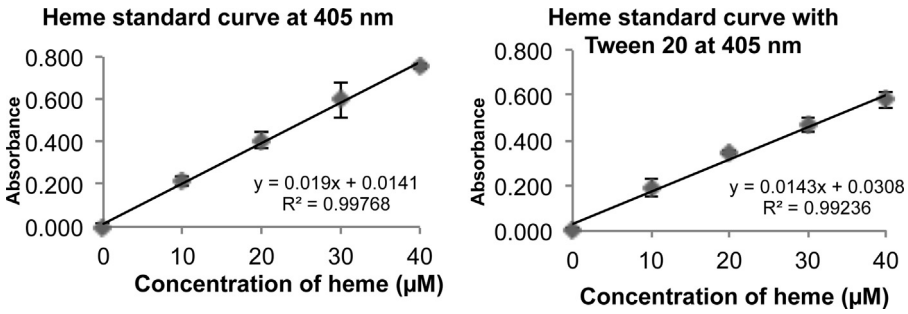

Fig. 1. (a) Standard curve of heme solution at 405 nm without activator (b) standard curve of heme solution with activator (tween 20) at 405 nm.

(PerkinElmer) and Lambda 25 UV spectrophotometer were used for analysis of heme.

Stock solution of hemin chloride was prepared by dissolving 10 mg of hemin chloride in 10 ml of DMSO (1533.7 μM). Hemin chloride being light sensitive, the solution preparation was carried out in dark and stored in plastic container protected from light at 4 °C. Since food vacuole of parasite is acidic, phosphate buffers of pH 5 and 5.5 were tested to get optimum formation of β-hematin from heme which showed clear solutions of heme over long period of time. Incubation of heme solutions in buffers (pH 5 and 5.5) for 2–6 days did not any show increase in absorbance at 405 nm. So there was negligible polymerization of heme observed in absence of activators. Tween 20, tween 80, oleic acid and stearic acid stock solutions (10 μM) were prepared in distilled water which were used as activators for β-hematin formation. Except stearic acid, the activators showed 80–90% conversion of heme to its polymerized form in buffer solutions. Tween 20 and 80 were selected as activators for further optimization of assay. A study of effect of concentrations of tween 20 and 80 (0.2–0.5 μM) on heme polymerization in presence of both buffers was carried out. Buffer pH 5.5 showed lower heme polymerization for all activators as compared to pH 5. Tween 20 showed maximum polymerization at all concentrations in presence of pH 5 buffer which was finalized for the assay.

There was a linear increase in absorbance observed when heme solutions (10-50 μM) were incubated in presence of tween 20 (0.5 μM). The heme polymerization was observed for 2-12 h time in presence of activator where maximum polymerization was observed in 1-2 h time. Hence, the optimized assay conditions involved solution of 30 μM heme, 0.5 μM tween 20, and phosphate buffer pH 5 to be incubated at 37 °C for 1-2 h. The absorbance of solutions was recorded at 405 nm and 630 nm against a blank solution without hemin chloride.

After development of spectrophotometric method, suitable changes in volume of assay solution were done to adopt the method for high throughput screening using 96 well plate reader. In multiwell assay for standard curve of heme, different concentrations (10-40 μM) of heme were dispensed in 96 well plate and the volume was made up to 200 μl with phosphate buffer pH 5, one set without adding the activator and the other with activator (tween 20). Concentration of heme was found to be linear over a range of 10–40 μM and in the absence and presence of tween 20 (activator) showed linearity in decrease in absorbance at 405 nm (heme degradation) (Fig. 1).

As reported in literature, Chloroquine (standard anti-malarial) acts on heme degradation in malarial parasite. For assay of chloroquine, hemin chloride (30 μM) was dispensed in a 96-well plate, which was followed by addition of chloroquine (1-6 μM solution in water) and the volume was made up with phosphate buffer pH 5 to 200 μl. It was then allowed to stand for 15 min after which tween 20 (0.5 μM) was added. The plate was protected from exposure to light and was incubated at 37 °C for 1 h. After 1 h, the absorbance was measured at wavelengths 405 nm and 630 nm. The assay was performed in triplicate and results were expressed as average ±SD. Concentration of heme in test or standard solutions remaining after incubation time was calculated from standard curve of heme.

For assay carried out at 405 nm, decrease in heme polymerization was calculated as follows -

$$\text{Heme conversion fraction} : \frac{(\text{Heme}_{-\text{T}} - \text{Heme}_{\text{Test}})}{(\text{Heme}_{-\text{T}} - \text{Heme}_{\text{T}})}$$

$$\%\text{inhibition of heme polymerizaion} : (1 - \text{heme conversion fraction}) \times 100$$

For assay carried out at 630 nm, decrease in heme polymerization was calculated as follows -

$$\text{Heme conversion fraction} : \frac{(\text{Heme}_{\text{T}} - \text{Heme}_{-\text{T}})}{(\text{Heme}_{\text{Test}} - \text{Heme}_{-\text{T}})}$$

$$\%\text{of control (heme polymerization)} = (1 - \text{heme conversion fraction}) \times 100$$

where Heme_{T}: Heme solution with tween 20, $\text{Heme}_{-\text{T}}$: Heme solution without tween 20, $\text{Heme}_{\text{Test}}$: Heme solution containing standard (chloroquine) or any test substance.

Heme polymerization was well inhibited by chloroquine (Fig. 2a and b) at low concentration which was detected at 405 and 630 nm, thus establishing utility of the assay procedure. The method was further used to check ability of primaquine (100-300 μM in water), quinine (10-50 μM in water) and artemether (300-1500 μM in DMSO-water) to inhibit heme polymerization. As expected, quinine inhibited heme polymerization at low concentration (Fig. 2c and d). Primaquine showed less affinity to bind to heme for its detoxification (Fig. 2c and d) and artemether showed least heme binding capability (Fig. 2e and f) as compared to other drugs under study. The method was then applied to synthetic compounds and extracts of medicinal plant for determining antimalarial activity (unpublished results). The developed heme polymerization method was also found to be useful for anti-malarial screening of variety of synthetic compounds and plant extracts specially due to use of two wavelengths.

The developed heme polymerization assay method was found to be precise as there was no isolation of polymerized heme. The assay procedure was simple, accurate and quick which showed reproducible results. The detection of heme polymerization was carried out at two wavelengths. Chloroquine and quinine which are known to be anti-malarial drugs acting on heme detoxification pathway were used as positive control in the method along with drugs like artemether and primaquine having limited action on heme degradation. The method could distinguish these drugs based on this mechanism of action. Spectrophotometric method for heme polymerization can be used for small number of samples and with simple instrumentation. HTS method without isolation of hematin and turbidity can be used for screening of multiple compounds. The