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Detection, quantification, and investigation of the red blood cell partitioning of cryptolepine hydrochloride

[Detección, cuantificación e investigación del reparto de clorhidrato de criptolepina en glóbulos rojos]

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Abstract

Context: The fight against malaria is limited by development of resistance of *Plasmodium* to medication. This has led to an urgent search for alternative medicinal agents.

Aims: To determine the affinity of the cryptolepine for the red blood cell.

Methods: HPLC method for the identification and quantification of cryptolepine was developed. Lipid solubility for both quinine (control) and cryptolepine was determined. Partitioning and repartitioning of cryptolepine into RBCs were studied. Time, concentration, temperature and pH were varied to see their effect on the partitioning of cryptolepine. Plasma protein binding was determined by the red blood cell partitioning method.

Results: An accurate, precise and robust HPLC method for cryptolepine hydrochloride was developed. Cryptolepine and quinine had lipophilicity of 0.91 ± 0.02 and 1.52 ± 0.27, respectively. The highest partitioning values of 2.02 ± 0.08 for cryptolepine and 0.93 ± 0.02 for quinine were obtained at 40 minutes. Concentration-dependent protein binding was observed for both compounds with cryptolepine having 0.43 and 0.38 for quinine. Partitioning was also found to be temperature dependent with the highest partitioning obtained at 37°C for cryptolepine (1.56 ± 0.04) and quinine (0.78 ± 0.01). Partitioning of cryptolepine and quinine were inversely related to pH with R^2 values of 0.94 and 0.96, respectively. P-values between partitioning and repartitioning for cryptolepine and quinine were 0.04 and 0.05, respectively.

Conclusions: Partitioning was found to be time, temperature, concentration and pH dependent. Partitioning was irreversible for cryptolepine and reversible for quinine. Protein binding in both cases was moderate.

Keywords: 4-aminoquinolone; erythrocyte; ion-trapping; quinine; repartitioning.

Resumen

Contexto: La lucha contra el paludismo está limitada por el desarrollo de la resistencia del *Plasmodium* a los medicamentos. Esto ha llevado a una urgente búsqueda de agentes medicinales alternativos.

Objetivos: Determinar la afinidad de la criptolepina para glóbulos rojos.

Métodos: Se desarrolló el método de HPLC para la identificación y cuantificación de criptolepina. Se determinó la solubilidad en lípidos tanto para la quinina (control) como para la criptolepina. Se estudió la división y repartición de la criptolepina en los glóbulos rojos. El tiempo, la concentración, la temperatura y el pH se variaron para ver su efecto en la partición de la criptolepina. La unión a proteínas plasmáticas se determinó mediante el método de división de glóbulos rojos.

Resultados: Se desarrolló un método HPLC exacto, preciso y robusto para el hidrocloruro de criptolepina. La criptolepina y la quinina tuvieron lipofilicidad de 0,91 ± 0,02 y 1,52 ± 0,27, respectivamente. Los valores de partición más altos de 2,02 ± 0,08 para criptolepina y 0,93 ± 0,02 para quinina se obtuvieron a los 40 minutos. Se observó unión a proteínas dependiente de la concentración para ambos compuestos con criptolepina 0,43 y 0,38 para quinina. El reparto también se encontró que dependía de la temperatura con la partición más alta obtenida a 37°C para la criptolepina (1,56 ± 0,04) y la quinina (0,78 ± 0,01). La división de la criptolepina y la quinina se relacionó inversamente con el pH con valores de R^2 de 0,94 y 0,96, respectivamente. Los valores p entre partición y reparticionado para criptolepina y quinina fueron de 0.04 y 0.05, respectivamente.

Conclusiones: Se encontró que la partición depende del tiempo, la temperatura, la concentración y el pH. La partición fue irreversible para criptolepina y reversible para quinina. La unión a proteínas en ambos casos fue moderada.

Palabras Clave: 4-aminoquinolona; atrapamiento de iones; eritrocito; quinina; reparto.

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INTRODUCTION

Malaria, a global health issue is a mosquito borne infectious disease caused by five species of the plasmodium parasite notably *P. falciparum, P. malariae, P. vivax, P. ovale* and *P. knwolesi*. At a point in history, it was a pandemic, but is now limited to the tropics where the vector (anopheles mosquito), thrives best (Hanson, 2005). The disease claims lives of all age groups. The more susceptible groups are children below five years, pregnant women and immigrants who have been in malaria free zones for more than three months. Globally, 214 million cases of malaria were reported for 2015, 438,000 deaths resulted from these cases. It is worth noting that, prevalence of the disease has decreased by 37% over the past 15 years according to the 2015 WHO malaria report (WHO, 2016). This can be attributed to the fact that, malaria is one of the diseases targeted for elimination by the Sustainable Development Goals hence a lot of attention has been given to it. A considerable challenge in the fight against malaria is the development of resistant strains, which limits the treatment options available. The parasite develops mechanisms to remain resistant to drugs used for treatment. This has led to an urgent search for alternative medicinal agents to fight the disease.

Plants have served as sources of lead compounds for synthetic and semisynthetic drugs. For malaria treatment, quinine and the artemisinins are examples of breakthrough compounds that were isolated from plants (Lavrado et al., 2010). Cryptolepine, an alkaloid isolated from the roots of *Cryptolepis sanguinolenta* (*Periplocaceae*) has shown remarkable antimalarial activity (Grellier et al., 1996). As an antimalarial, it is chemically related to the 4-aminoquinoline compounds, which work by attacking the erythrocytic stage of the plasmodium parasite's development in the human host. The 4-aminoquinolines inhibit the conversion of heme to hemozoin, this leads to the accumulation of heme, which causes lyses of the parasite (Kaschula et al., 2002). The erythrocytic stage of the parasites development is responsible for the signs and symptoms related to malaria (Rosenthal et al., 1988). Therefore, a remedy to halt the erythrocytic stage of the parasite will prevent the clinical manifestations of the disease, thereby preventing complications and reducing mortality. Little has been reported on the concentration of cryptolepine at its proposed site of action (food vacuole of the parasites within the RBCs). This study therefore aims to determine the affinity of cryptolepine for the red blood cell (RBC) and some factors that may affect its accumulation at its target site.

MATERIAL AND METHODS

Materials

The cryptolepine hydrochloride sample used in the study was isolated and characterized in an earlier study (Kuntworbe et al., 2012). Diazepam and quinine powders were obtained from Ernest Chemist Limited, Ghana. HPLC grade acetonitrile and methanol (Sigma) were acquired from the Histamine laboratory at Ghana Standards Authority and the Food Science Mycotoxin laboratory, Knust. Formic acid (Sigma) was obtained from Noguchi Memorial Institute, for Medical Research, Legon. Ammonium acetate (Fizmerk) was purchased from Paveboa Ventures, Accra. Red blood sample was collected from a blood bank (Fanteakwa District Hospital, Begoro, Ghana).

HPLC development

The HPLC system for cryptolepine assay comprised of a Shimadzu Prominence UFLC system (Shimadzu Incorporation, Tokyo, Japan) with LC–20A quaternary pump, DGU-20A_5 in-line vacuum degasser and SPD-20A UV detector. The chromatographic separation was carried out on a C18 column, Perkin Elmer with dimensions 4.6 x 250 mm and a pore size of 5 µm, 100 Å. Data acquisition was by the LC solutions software version 125 (Shimadzu Incorporation, Tokyo, Japan). Diazepam was used as the internal standard.

An isocratic elution consisting of 35% phase A (water adjusted to a pH of 2.5 with formic acid) and 65% phase B (acetonitrile) was considered suitable. This mobile phase system produced peaks with good resolution, symmetry, distinct retention times and stable baseline. The column was kept at constant temperature of 25°C in a column oven; the detector was set to a wavelength of 282 nm for

identification and quantification of analyte and internal standard (diazepam) at a solvent flow rate of 1.0 mL/min. The injection volume was 20 µL with a run time of 10 minutes. The peaks were distinguished by their retention times.

The method was validated based on the International Conference on Harmonization Guidelines on linearity, precision and accuracy (ICH, 2005).

For quinine an already developed and validated HPLC method (Kolawole and Mustapha, 2000) was used. The HPLC system consisted of a Shimadzu RF-10A$_{XL}$ fluorescence detector, Cecil Adept CE 4600 column oven, Cecil Adept CE 4800 pump in line with a degasser and a C18 column, Phenomenex with dimensions 100 x 4.6 mm and a pore size of 5 µm 100 A. Data Acquisition was by PowerStream.

Lipophilicity

Cryptolepine hydrochloride (0.5 mg) was added to a mixture of equal volumes of water and pentanol (2 mL each) in a separating funnel, which was then clamped and allowed to stand for 20 minutes. Each solvent (200 µL) was pipetted and extraction carried out using a previous approach (Kuntworbe, 2012). The amount of analyte was quantified by their respective HPLC methods.

RBC partitioning assay

Determination of the effect of time on Ke/p

The traditional method of RBC partitioning was used (Hinderling, 1997). Blood spiked with cryptolepine to a concentration of 800 µg/mL was left at 37°C for 2 hours in a water bath set to oscillate at 20 oscillations per minute. At 0, 10, 20, 40, 60, 90 and 120 minutes, 1 mL blood was pipetted into an eppendorf tube and centrifuged at 6500 *xg* for 5 minutes. Plasma (200 µL) was pipetted into 1.6 mL of chilled acetonitrile and cryptolepine was extracted. RBCs (200 µL) was pipetted and lysed in distilled water (800 µL), followed by extraction of cryptolepine in chilled acetonitrile. Extracts were kept at -20°C in an ultra-low temperature freezer until analyzed by the validated HPLC method. Before assay, diazepam was added to obtain a concentration of 3.75 µg/mL (Hinderling, 1997). The RBC partitioning in whole blood (Ke/p) was determined using the equation below:

$$Ke/p = Ce/Cp \quad [1]$$

Where Ce is concentration of analyte in red blood cell; Cp is concentration of analyte in plasma.

Determination of the reversibility of Ke/p of cryptolepine

RBCs were separated from plasma by centrifuging at 10,000 *xg* for 5 minutes. Cryptolepine was added to RBCs before plasma was added to obtain cryptolepine concentration of 800 µg/mL. At 0, 10, 20, 40, 60, 90 and 120 minutes, 1 mL blood was pipetted into an eppendorf tube and centrifuged at 6500 *xg* for 5 minutes. Plasma (200 µL) was pipetted and cryptolepine extracted in 1.6 mL chilled acetonitrile. RBCs (200 µL) were pipetted and lysed in 800 µL water. Chilled acetonitrile (800 µL) was then added for extraction. Plasma and RBCs extracts were centrifuged at 6500 *xg* for 5 minutes to allow precipitate to settle and kept at -20°C in an ultra-low temperature freezer till it was assayed. The value for repartitioning obtained at different times was compared to that of partitioning.

Determination of the effect of concentration on RBC partitioning

Partitioning experiment was conducted as described above at different concentrations. Whole blood samples were spiked with working stock cryptolepine to obtain concentrations (400, 800, 1000 and 1500 µg/mL). The tubes were then allowed to stand for 2 hours at 37°C in an oscillating water bath. The spiked whole blood was centrifuged at 6500 *xg* for 5 minutes. Plasma (200 µL) was pipetted for extraction of cryptolepine using 1.6 mL chilled acetonitrile. RBCs (200 µL) were lysed in 800 µL water, and extraction of cryptolepine carried out in 800 µL acetonitrile. The extract was kept at -20°C in an ultra-low temperature freezer until analyzed.

Determination of effect of concentration on protein binding of cryptolepine

The protein binding experiment was carried out at different concentrations of spiked whole blood and corresponding RBC in PBS. Whole blood and RBC suspensions were spiked with working standard cryptolepine to obtain concentrations of (400, 800, 1000 and 1500 µg/mL). After incubating in the

oscillating water bath at 37°C, the spiked whole blood and their corresponding RBC suspension were centrifuged at 6500 *xg* for 5 minutes. Plasma (200 µL) was added to chilled acetonitrile to precipitate proteins and extract cryptolepine. RBCs (200 µL) were lysed in water (800 µL) and cryptolepine extracted in acetonitrile. The extracts were kept at -20°C in an ultra-low temperature freezer until analyzed (Hinderling et al., 1984). The parameters were deduced from the equations below:

$$Ke/p.u = Ce/Cp.u \quad [2]$$

$$Ke/p = Ke/p.u.fu \quad [3]$$

$$fu = (Ke/p)/(Ke/p.u) \quad [4]$$

$$fb = 1\text{-}fu \quad [5]$$

Where Ke/p.u, is the RBC partitioning in phosphate buffered saline; Cp.u, concentration of drug in buffer; Ke/p, RBC partitioning in whole blood; fu, fraction of unbound drug; fb, fraction of bound drug.

Determination of effect of pH on RBC partitioning

Partitioning experiment was conducted by the traditional method at different pH values. Six eppendorf (Eppendorf, Hamburg, Germany) tubes each containing 700 µL of blood were centrifuged at 10000 g for 5 minutes. Plasma was pipetted out and replaced with equal volume of PBS adjusted to pHs of 2, 4, 6, 8, 10 and 12. To each tube, cryptolepine solution was added to a concentration of 800 µg/mL. After incubating for 1 hour, the spiked whole blood was centrifuged at 6500 *xg* for 5 minutes. Cryptolepine from pH adjusted PSB (200 µL) was extracted in 1.6 mL chilled acetonitrile. RBCs (200 µL) were lysed in 800 µL water, and extraction of cryptolepine carried out in 800 µL acetonitrile. The extracts were kept at -20°C in an ultra-low temperature freezer until analyzed.

Determination of the effect of temperature on RBC partitioning

Three eppendorf tubes containing whole blood spiked with 800 µg/mL cryptolepine were left to stand for 1 hour at different temperatures (25, 37 and 40°C) in the oscillating water bath. After incubation, 200 µL plasma was extracted in 1.6 mL chilled acetonitrile. RBCs (200 µL) was lysed in 800 µL water and extraction of cryptolepine carried out in 800 µL acetonitrile. The extract was kept at -20°C in an ultra-low temperature freezer until analyzed (Fisar et al., 2006). RBC partitioning experiments were repeated for quinine (control) at stated times, concentrations, temperatures and pHs

Statistical analysis

Results were expressed as mean ± SD. Data was derived by Microsoft Excel 2010 except the determination of level of significance, which was done by Graph Pad Prism Version 6 (GraphPad Sofware, California, USA) at confidence interval of 95%.

RESULTS

Validation parameters

The HPLC method was robust with a peak retention time of 5.37 minutes for cryptolepine (Fig. 1). The peak retention time for the control drug quinine was 3.54 minutes (Fig. 2).

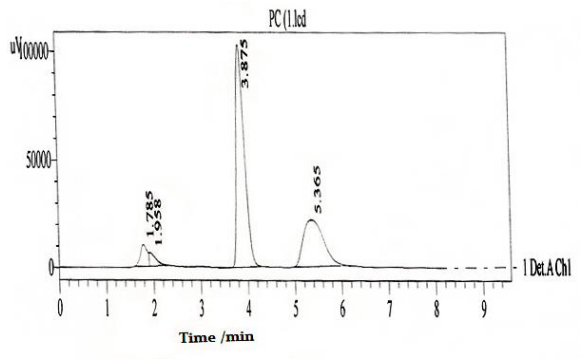

Figure 1. Chromatogram of cryptolepine (with retention time 5.37 minutes) and that of diazepam (retention time 3.88 minutes. The diazepam was used as the internal standard.

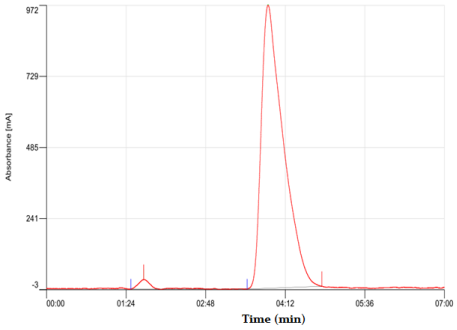

Figure 2. Chromatogram of quinine (with retention time of 4.12 minutes).

The HPLC method showed good correlation between cryptolepine concentration and peak area (Fig. 3). The limits of detection and quantification were 4.06 and 12.31 µg/mL, respectively with %RSDs less than 2% for both inter-day and intra-day determinations. The percentage recoveries of the extraction process were 98.96 ± 0.21% and 97.54 ± 0.48% at low and high cryptolepine concentrations. There was also good correlation between concentration of quinine and peak area (Fig. 4). The percentage recoveries for the extraction process for quinine were 89.29 ± 3.58% and 88.42 ± 2.41% at low and high quinine concentrations.

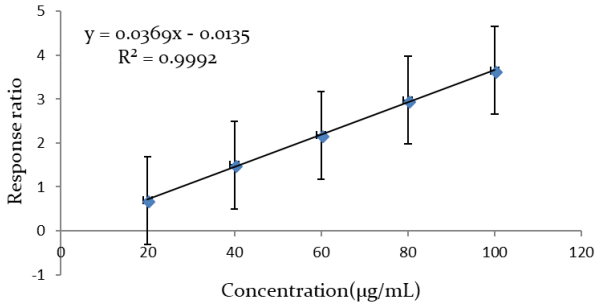

Figure 3. Linearity curve of response ratio against cryptolepine concentration. The R^2 value 0.9992 indicates a near perfect correlation between the response ratio and concentration of cryotplepine.

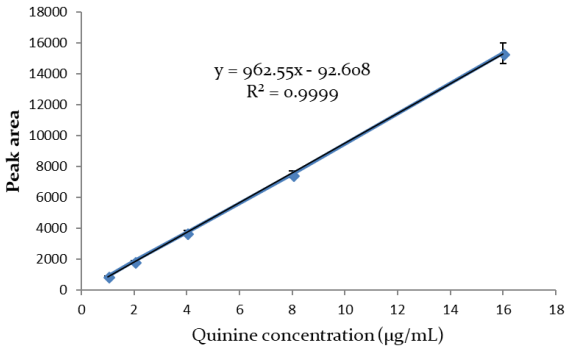

Figure 4. Linearity curve of peak area against quinine concentration there is a perfect relationship between quinine concentration and peak area.

Lipophilicity

Pentanol-water partitioning for cryptolepine was 0.9 ± 0.02, which is significantly different ($p<0.001$) from the value for quinine (1.52 ± 0.27).

RBC partitioning

The Ke/p values at different time-point are given in Table 1. For cryptolepine, the highest Ke/p was 2.02 ± 0.08 at 40 minutes. P value between red blood cell partitioning and repartitioning for cryptolepine was 0.004. For quinine, the highest Ke/p was 0.93 ± 0.02 at 40 minutes and the p value between partitioning and repartitioning was 0.005.

Ke/p and Ke/p.u were proportional to concentration for both cryptolepine and quinine (Table 2). Generally, Ke/p values were greater than Ke/p.u. Fraction of cryptolepine bound to plasma protein was 0.43 ± 0.04 whilst that of quinine was 0.38 ± 0.14. These values were not significantly different ($p<0.05$).

Ke/p was found to be inversely proportional to pH. There was good correlation between pH and Ke/p values for both cryptolepine and quinine (Table 3).

Varying temperature, the lowest Ke/p was observed at 25°C and the highest at 37°C for both cryptolepine and quinine.

Temperature appears to have significantly different ($p<0.001$) effect on the concentrations of Cryptolepine and Quinine in both plasma and RBCs (Table 4).

DISCUSSION

The pentanol-water partitioning of quinine and cryptolepine showed quinine to be more lipophilic. The more lipophilic a compound is, the less its aqueous solubility. The low aqueous solubility of Quinine sulphate was in conformity with that reported by Strauch et al. (2011). Because most drugs are either weakly acidic or basic in nature, they mostly have low aqueous solubility. This calls for the addition of counter-ions to form salts with improved physicochemical and biopharmaceutical properties (especially in terms of aqueous solubility). HCl and SO_4^{2-} were counter ions attached to the skeleton structures of cryptolepine and quinine respectively. The diprotonic nature of H_2SO_4 gives HSO^-_4 and H^+ on initial ionization. HSO^-_4 anion is also acidic but with less ability to dissociate into H^+ and SO^-_4 than either H_2SO_4 or HCl, hence its second

dissociation is not normally considered. HCl allows complete ionizations into H^+ and Cl^- making HCl a stronger acid as compared to H_2SO_4. The complete ionization for HCl generates ions, which are more polar as compared to ions from H_2SO_4 causing cryptolepine hydrochloride to be more water soluble than quinine sulphate.

Again, for a successful saltation, the pKa difference between a basic drug and its counter-ion should be a minimum of 2 (Ball et al., 1948). The latter should be at least 2 pHs below the pKa of the former (Stahl and Wermuth, 2002). Quinine is a dibasic drug with pKa of 8.5 and 4.1 (Salako and Sowunmi, 1992) and sulphuric acid has a pKa of -3 (Niaz and Maza, 2011) creating a pH difference of 11.5 and 8.1. Cryptolepine and hydrochloric acid have pKa of 10.99 (Kuntworbe et al., 2013) and -6.3 (Niaz and Maza, 2011), creating a pH difference of 13.99. The bigger the difference, the better the salt formed, this makes cryptolepine hydrochloride a better salt hence more water soluble.

The lipoid nature of the membrane of the RBCs allows a lipid soluble drug to penetrate easily, however a tint of solubility in aqueous phase is required to permit the dissolution of the drug in plasma water from where it gets to the cells of the blood for penetration. Sufficient aqueous solubility and moderate lipophilicity help chemicals rapidly diffuse through lipid and aqueous phases. Quinine with low water solubility will have difficulties dissolving in plasma water as compared to cryptolepine.

Table 1. Effect of time on red blood cells partitioning.

Time (mins)	Partitioning values		Repartitioning values	
	Ke/p quinine	Ke/p cryptolepine	Ke/p quinine	Ke/p cryptolepine
5	0.61 ± 0.004	1.57 ± 0.03**	0.70 ± 0.02	1.72 ± 0.17***
10	0.65 ± 0.05	1.37 ± 0.06ns	0.60±0.0005	1.55 ± 0.12***
20	0.64 ± 0.006	1.28 ± 0.08ns	0.58 ± 0.02	1.97 ± 0.09***
40	0.93 ± 0.02	2.02 ± 0.08**	0.44 ± 0.05	1.89 ± 0.14***
60	0.78 ± 0.005	1.56 ± 0.04*	0.41 ± 0.04	1.72 ± 0.006***
90	0.70 ± 0.03	1.55 ± 0.06*	0.41 ± 0.003	1.98 ± 0.25***
120	0.73 ± 0.05	1.03 ± 1.01ns	0.59 ± 0.006	1.64 ± 0.08***

The data in this Table represent the mean ± SD (n=3) of the partitioning and repartitioning of cryptolepine and quinine into and out of RBCs in whole blood at different time points. The mean values of Ke/p of cryptolepine for partitioning and repartitioning compared to those of quinine statistically are given by the notations as follows: *p<0.05; **p<0.01; ***p<0.001; nsp>0.05.

Table 2. Effect of concentration on Ke/p, Ke/p.u and fraction bound(fb).

Conc. (µg/mL)	Ke/p quinine	Ke/p cryptolepine	Ke/p.u quinine	Ke/p.u cryptolepine	(fb) quinine	(fb) cryptolepine
400	0.68 ± 0.003	0.76 ± 0.004ns	0.91 ± 0.05	1.05 ± 0.54ns	0.25 ± 0.27	0.28 ± 0.12ns
800	0.78 ± 0.02	1.64 ± 0.02***	1.12 ± 0.06	2.34 ± 0.07***	0.30 ± 0.65	0.43 ± 0.04ns
1000	0.79 ± 0.06	1.58 ± 0.02***	1.14 ± 0.04	2.64 ± 0.15***	0.31 ± 0.04	0.40 ± 0.02ns
1500	0.81 ± 0.12	1.95 ± 0.03***	1.30 ± 0.06	3.18 ± 0.02***	0.38 ± 0.14	0.39 ± 0.05ns

The data represent the mean ± SD (n=3) of the partitioning of cryptolepine and quinine into RBCs in whole blood (Ke/p) and RBCs suspended in phosphate buffer saline (Ke/p.u) and fraction of drug bound to plasma protein (fb) within a drug concentration range of 400-1500 µg/mL. At 400 µg/mL, the Ke/p, Ke/p.u and fb of cryptolepine were similar to those of quinine. Above 400 µg/mL however the values were statistically different as given by the notations: ***p<0.001; nsp>0.05.

Table 3. Effect of pH on RBC partitioning of cryptolepine and quinine.

pH	Ke/p quinine	Ke/p cryptolepine
2	1.41 ± 0.02	1.23 ± 0.01***
4	1.26 ± 0.003	1.16 ± 0.04**
6	1.11 ± 0.08	1.04 ± 0.05ns
8	0.93 ± 0.01	0.94 ± 0.02ns
10	0.95 ± 0.02	0.59 ± 0.08***
12	0.67 ± 0.02	0.54 ± 0.01***
R^2		0.94

The data represent the mean ± SD (n=3) of the partitioning of cryptolepine and quinine into RBCs in whole blood (Ke/p) within the pH range of 2-12. Below pH 6 and above pH 8, the Ke/p values of cryptolepine are statistically different from those of quinine. The statistical notations are as follows: **p<0.01; ***p<0.001; nsp>0.05.

Table 4. Effect of temperature on red blood cells partitioning of cryptolepine and quinine.

Temperature (°C)	Ke/p quinine	Ke/p cryptolepine
25	0.45 ± 0.01	1.29 ± 0.04***
37	0.78 ± 0.02	1.56 ± 0.17***
40	0.23 ± 0.008	1.51 ± 0.05***

The data represent the mean ± SD (n=3) of the partitioning of cryptolepine and quinine into RBCs in whole blood (Ke/p) within the temperature range of 25-40oC. the Ke/p of cryptolepine were statistical different (***p<0.001) from those of quinine within the temperature range.

RBC partitioning assay

It is advisable to initially study the rate of partitioning of drug to predict when steady state of equilibrium is reached so that the extent of RBC partitioning can be appropriately estimated (Hinderling, 1997). The partitioning study was done under various conditions to establish the extent to which the partitioning is affected by those conditions.

Effect of time on partitioning and reversibility of partitioning

The partitioning of cryptolepine into the RBC was found to be time dependent (Table 1). Ke/p decreased from 1.57 to 1.28 for the first 20 minutes. In an unpublished work (Efficacy, pharmacokinetics and safety of cryptolepine-artemisinins based combination in the management of malaria), aldehyde oxidase, human specific UDP-glucuronosyltransferase (UGT) and cytochrome P450 were the main enzymes involved in the metabolism of cryptolepine in rat and human (Forkuo et al., 2017), but the presence of these enzymes in the RBCs has not been proven scientifically. These enzymes can be the possible cause of decrease in cryptolepine levels that initially entered the cell. At 40 minutes, the highest partitioning of 2.02 was observed for cryptolepine. The drastic increase was due to the fact that, the fraction of drug bound to plasma proteins served as a reservoir to keep the equilibrium state between plasma and tissue concentrations as degradation occurred in the cell. From time 60-120 minutes, partitioning decreased from 2.02 to 1.03. At 2 hours, it can be said that equilibrium concentrations of cryptolepine in plasma and RBCs had been attained. It can also be inferred that, cryptolepine has greater affinity for the RBCs in relation to plasma as was expected from the lipophilicity studies. With time, the Ke/p of quinine had no particular pattern, over the two hour period, partitioning increased and decreased as time passed by, this is very similar to the partitioning obtained for formononetin (Singh et al., 2011). Highest Ke/p of 0.93 was observed at 40 minutes. The presence of amino groups in both quinine and cryptolepine is most likely responsible for the delay in attaining a steady state (Hinderling, 1997). Bumetanide, gentamycin methotrexate, vancomycin and procainamide, with primary amino groups have exhibited delay in attaining steady state concentrations between plasma and RBCs.

The repartitioning assay was done to check the reversibility of the partitioning of cryptolepine into RBCs. Significant difference between partitioning and repartitioning values was determined by graph pad prism version 6 using the paired T-test between a confidence interval of 95%. P-value less than 0.05 shows a significant difference between sets compared (Hinderling, 1997). Partitioning and repartitioning p-values were of 0.05 and 0.04 for quinine and cryptolepine respectively. P-value less than 0.05 shows a significant difference between sets compared (Hinderling, 1997). There was significant difference between the partitioning and repartitioning

values for cryptolepine suggesting a degree of irreversibility in the binding of cryptolepine to the RBC. Cryptolepine (100 mg/kg) caused a significant decrease in mean corpuscular volume as compared to 25 and 50 mg/kg in a study by Forkuo et al. (2016), and the irreversibility of the binding can be responsible for this decrease. Cryptolepine is likely to have a dose dependent toxicity on red blood cells.

Effect of concentration on Ke/p, Ke/p.u and protein binding

The RBC partitioning was observed to be concentration dependent (Table 2). Concentration dependent partitioning suggests that the uptake of cryptolepine by the cell is not only by passive diffusion but other factors such as protein binding, ion trapping and active transport may be involved (Wallace and Riegelman, 1977). For Ke/p values higher than 2, extrapolating pharmacokinetics parameters from plasma will be misleading, hence whole blood concentration of drug will be more convenient in such a case (Hinderling, 1997). At cryptolepine concentrations of 800, 1000 and 1500 µg/mL, Ke/p was 1.64, 1.58 and 1.95, respectively. Ke/p values above 1 indicates higher affinity of cryptolepine for the RBC than plasma. At cryptolepine concentration of 400 µg/mL the Ke/p was less than 1 (0.76). The partitioning can be said to be directly proportional to concentration. At higher concentration, the plasma proteins responsible for binding might be saturated and this allows free cryptolepine to enter the cell. RBC partitioning of quinine was also found to be concentration dependent within the same concentration range studied with Ke/p less than 1.

Partitioning was also studied in a suspension of RBC in PBS at pH 7.4 (Table 2). Partitioning in the absence of plasma protein (Ke/p.u) can be explained as a measure of the complete affinity of drug to the binding sites in the RBCs. Ke/p.u also showed concentration dependent partitioning and was directly proportional to concentration. At cryptolepine and quinine concentrations of (400, 800, 1000 and 1500 µg/mL) partitioning coefficient of (1.05, 2.34, 2.64 and 3.18) and (0.91, 1.11, 1.14 and 1.26) were obtained, respectively. The partitioning of RBCs in PBS was higher than that in whole blood. This can be attributed to the fact that, PBS unlike plasma lacks proteins for binding and metabolism. It also buttresses the fact that protein bound drugs are not available for partitioning hence the decrease in the partitioning in whole blood as compared to partitioning in PBS (Ehrnebo and Odar-Cederlöf, 1977). In the absence of plasma proteins, the partitioning coefficient obtained were all above 1 for cryptolepine and quinine except for quinine at 400 µg/mL. This means that, in the absence of plasma proteins, cryptolepine and quinine have greater affinity for the RBCs than that of plasma. Dosing of cryptolepine and quinine in medical conditions where plasma protein is reduced should consider the fact that RBC accumulation of both drugs can occur.

Protein binding is influenced by the physicochemical properties of the drug, the total concentration of the drug in the body, the physicochemical nature of the protein synthesized the amount of protein available, the affinity between drug and protein, and drug interactions (Jusko and Gretch, 2008). Plasma protein binding can be determined by equilibrium dialysis, ultrafiltration and RBC partitioning assay (Bowers et al., 1984). For equilibrium dialysis and ultrafiltration, protein binding is experimented in the absence of the cells of the blood. This makes the method inaccurate because the binding of drugs to the cells of the blood is also a form of protein binding. Plasma protein binding can be mathematically deduced from the values of Ke/p and Ke/p.u. Protein binding initially increased for cryptolepine from 0.28 to 0.43. Fraction bound decreased slightly from 0.40 to 0.39 as cryptolepine concentration increased from 1000 to 1500 µg/mL. At higher cryptolepine concentrations, plasma proteins are likely to be saturated, causing the fall in fraction of protein bound by cryptolepine from 1000 to 1500 µg/mL. At higher concentrations, a lot of cryptolepine will be free and available for activity and distribution. Comparing protein binding of cryptolepine by RBC partitioning with that obtained from equilibrium dialysis (0.58) in an unpublished work carried out (Efficacy, pharmacokinetics and safety of cryptolepine-artemisinin based combinations in the management of malaria), protein binding of cryptolepine can be said to be moderate. The same study showed a very high volume of distribution (Vdss) for cryptolepine. The insignificant binding to protein, makes the free drug available for distribu-

tion into other cell compartments, hence the large Vdss.

Quinine had a protein binding ranging from 0.25 to 0.38 for the range of concentrations studied. Protein binding of quinine has been found to be dependent on concentration and the amount of alpha-1-glycoprotein present. quinine has been found to have a higher affinity for alpha-1-glycoproteins. This plasma protein constitutes just about 1-3% of the total plasma protein. This accounted for the low binding values obtained for quinine. quinine has been reported to have protein binding of between 49-69% (Silamut et al., 1991). This range was not obtained in the current study. It can be said that, the concentration range within which the study was conducted was low.

Effect of pH on Ke/p

Plasma has a pH of 7.40 and cytosol of RBC has a pH range between 7.10-7.30 (Hinderling, 1984). cryptolepine being basic in nature also has a pKa of 10.99 (Kuntworbe, 2012). The slight difference in pH is accountable for the passage of slightly electrolytic drug into the RBC from plasma. Acidic conditions favor the ionization of cryptolepine. The few unionized species readily cross the lipid membrane of the cell due to its lipophilicity. On entering the cell, the unionized group gets ionized and trapped in the RBC's compartment. Ionization contrarily prevents the translocation from the cell because it is not diffusible. For the stability of the cell, the unionized species try to be in equilibrium. This reverts some ionized species in the extracellular compartment to the unionized form which can easily cross the membrane into the compartment. It was observed that, decreasing pH of the PBS increased the RBC partitioning of cryptolepine. This can be attributed to the fact that ion trapping was occurring. Changing pH caused a change in the fraction of ionized molecules hence a change in permeability and partitioning.

Again, the lower Ke/p values at higher pH can be said to result from the fact that cryptolepine is more unstable in basic medium compared to acidic medium (Kuntworbe, 2012). The low partitioning of cryptolepine in basic medium can be attributed to the fact that a lot of cryptolepine had broken down, making it unavailable for partitioning. Ke/p of cryptolepine and quinine were found to be inversely related to pH with R^2 values of 0.94 and 0.96 respectively. The Ke/p values for quinine were generally higher than that of cryptolepine when pH was varied. This is because; changes in pH can affect the lipophilicity and aqueous solubility of a drug. It however also followed the same trend as cryptolepine; a decrease in pH caused an increase in RBCs partitioning.

In medical conditions, where the mechanisms in charge of controlling the body's pH are impaired, modifications should be done to the dosing of cryptolepine to avoid overdosing and under-dosing depending on the prevailing condition. For acidosis, the dose for cryptolepine and quinine can be decreased to prevent accumulation in the RBCs. For alkalosis, the dose can be increased since a lot of cryptolepine and quinine will be broken down under basic condition.

Effect of temperature on Ke/p

According to the first law of Ficks, passive diffusion is directly related to the diffusion coefficient (D), which is compound specific. D is dependent on a lot of variables including temperature. Hence, according to Ficks, the transport of a drug should increase as temperature increases. This was observed for cryptolepine and quinine at temperature 25°C and 37°C. Ke/p for cryptolepine increased from 1.29 to 1.56 at 25°C and 37°C, respectively. Ke/p for quinine also increased from 0.45 to 0.78 at 25°C and 37°C, respectively. The increase in Ke/p with increasing temperature can be attributed to the fact that passive diffusion is involved in the transportation of drugs into the RBCs. The lower Ke/p values at 25°C can also be attributed to the fact that, at this temperature, the proteins in charge of opening channels for uptake had reduced activity. At the optimum body temperature of 37°C, the highest Ke/p was observed for both cryptolepine and quinine. It can be said that, this is the optimum temperature for the protein in charge of uptake to function effectively. The low aqueous solubility of quinine also resulted in the lower Ke/p values for quinine in comparison to cryptolepine. It was however observed that, at 40°C, Ke/p for both cryptolepine (1.51) and quinine (0.23) decreased. This can be attributed to the fact that there was a lot of cell lysis

at 40°C as reported by Gershfeld and Murayama (1988), who studied the thermal instability of RBC membrane, they concluded that, above 37°C, hemolysis rates are rapid and are accompanied by gross changes in cellular morphology. Due to the delicate nature of the cells, intact cells available were not enough hence the decreased partitioning at 40°C. The proteins of the few intact cells available might be denatured at this temperature, making them unavailable for drug uptake. From Table 4, it can be observed that the concentrations of cryptolepine and quinine in both plasma and RBCs decreased drastically at 40°C. About 49 % decrease in cryptolepine concentration was reported when accelerated stability studies was conducted at 60°C (Kuntworbe, 2012). The stability of both drugs can therefore be said to be temperature dependent.

In medical conditions where body temperature rises, cryptolepine and quinine concentrations can be increased since its uptake is concentration dependent, to allow the few available cells to get enough cryptolepine to take up.

CONCLUSIONS

The HPLC method developed for cryptolepine was precise, robust, linear and specific. Ke/p for cryptolepine was irreversible with the highest Ke/p being 2.02 ± 0.008 at 40 minutes. Ke/p for cryptolepine was time, concentration, pH, and temperature dependent. Ke/p for quinine was reversible with the highest Ke/p being 0.93 ± 0.02 at 40 minutes. Ke/p for quinine was also dependent on time, concentrations, pH and temperature. Plasma protein binding of cryptolepine can be said to be moderate.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Concepts or ideas	X	X		
Design	X	X		
Definition of intellectual content	X	X		
Literature search	X			
Experimental studies	X			
Data acquisition	X			
Data analysis	X			
Statistical analysis	X			
Manuscript preparation	X	X		
Manuscript editing		X	X	X
Manuscript review	X	X	X	X

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