CHAPTER V

DISCUSSION

DHFR is the enzyme which catalyzes the reduction of dihydrofolate to tetrahydrofolate, having NADPH as a cofactor (Futterman, 1957). Being known that pyrimethamine is an inhibitor of DHFR (Hitchings and Burchall, 1965), thus, the study on binding properties between DHFR and pyrimethamine could be determined by the inhibitory effect of pyrimethamine to DHFR activities.

DHFR used in the research project was purified from rat liver by the method of McCoullough et al. (1971). The method proved to be suitable to eliminate those contaminating substances that might interfere the studies reaction. Some properties of DHFR from rat liver had been observed. The optimum condition for inhibition of pyrimethamine to DHFR was established at the pH range of 6.0-6.5 (Figure 3a and 3b) at $37^{o}C$. The stoichiometric inhibition of pyrimethamine to DHFR was demonstrated at pH 5.5, 6.0 and 7.5 in three different kinds of buffers. The complete inhibition of 2.81 units DHFR from rat liver in the above buffers had been observed to be the same at pyrimethamine concentration of 11.3×10^{-7} M (Figure 4). Since ethanol was used as the solvent for the extraction of pyrimethamine from liver and red blood cells of mice, the effect of ethanol on DHFR

activity was studied. It was clearly shown that ethanol could directly reduce the enzyme DHFR activity, especially at the higher concentration. When the amount of ethanol added to the incubation mixture was increased to 200 ul, the solution became turbidity due to colloidal proteins formation (Figure 5).

Various methods for the determination of pyrimethamine have been established in literatures, these include microbiological method (Hitchings, 1952; Weidekamm,et al., 1982), spectrophotometry (Schmidt,et al., 1953), thin-layer chromatography (TLC) (Jones and King, 1968; DeAngelis, et al., 1975; Admad and Rogers, 1980), gas chromatography (GC) (Cala, et al., 1972; Jones, et al., 1981; Bonini, et al., 1981) and high-performance liquid chromatography (HPLC) (Jones and Ovenell, 1979; Timm and Weidekamm, 1982; Edstein, 1984). However, there are some disadvantages among those analytical methods. The spectrophotometric method has no specificity and low sensitivity. The analysis utilizing TLC, GC, microbiological method and HPLC have to employ complicated procedures, time consuming and some methods need expensive instruments.

We have developed a highly specific, rapid and sensitive assay technique which is suitable to use for the determination of pyrimethamine in body fluids and tissues. The method is based on the competitive binding between ^{14}C-pyrimethamine/pyrimethamine and DHFR. The principle is modified from the competitive binding assay which has been developed and used for the determination of methotrexate

(DHFR inhibitor) which is widely used as antineoplastic and immunosuppressive agent (Myers, et al., 1975).

Several factors involved in the competitive binding assay procedures had been optimized. At the beginning the method was planned to use charcoal slurry, a known adsorbent of folates (Waxman and Schruber, 1973), as the adsorption substance to separate free pyrimethamine from the bound pyrimethamine-DHFR. Results from figure 6 indicated that over 98% of pyrimethamine concentration 2×10^{-5} M in the incubation mixture was removed by the charcoal at the concentration of 1.5 mg/ml in the incubation mixture. The procedure used for the subsequent competitive binding assay was designed to use 1.5 mg charcoal as the separation agent for free pyrimethamine.

The optimum time and temperature for the binding between ^{14}C-pyrimethamine and DHFR were also studied at 4^{o}C and 24^{o}C. Efficiency of binding between the pyrimethamine and DHFR at lower temperature (4^{o}C) was almost two times higher than that at 24^{o}C. The binding was rather steadily at various time of incubation up to 120 min at 4^{o}C (Figure 7). Hence, the incubation time of 15 min at 4^{o}C was selected as the condition for the standard assay method.

The linear correlation of DHFR concentration and binding capacity to ^{14}C-pyrimethamine indicated the possible use of the binding assay method at the presence of binding rat liver DHFR as high as 24 units. At this concentration, percent bound of pyrimethamine was about 50% (Figure 8).

The concentration of 10 nM ^{14}C-pyrimethamine was found to be appropriate to gain highest binding to 23.7 units of DHFR at the standard assay condition (Figure 9).

As it is known that DHFR catalyzes the conversion of dihydrofolate to tetrahydrofolate only in the presence of coenzyme NADPH, no conversion could be detected without this accessory compound. The results in figure 10 also indicated that binding of pyrimethamine to rat liver DHFR was absolutely dependent on NADPH. The maximum binding of pyrimethamine to DHFR (50%) was observed at 0.06 nM NADPH at the standard assay condition.

The result in figure 11 indicated optimum pH for binding of ^{14}C-pyrimethamine and DHFR in the range between pH 6.5-7.0. The data obtained was coincided to the value of optimum pH for the inhibitory effect of pyrimethamine on DHFR activity illustrated in figure 3. Thus, potassium phosphate buffer, pH 6.5 was selected as the buffer for the standard assay method.

The standard method developed allowed quantitative separation of bound pyrimethamine-DHFR complex from free pyrimethamine. Thus, it was made possible to calculate the association constant (K_a) of the pyrimethamine and rat liver DHFR at optimum conditions for binding. A Scatchard analysis (Scatchard, 1949) of pyrimethamine-DHFR binding, performed at pH 6.5, 4^{o}C and NADPH concentration of 0.1 mM indicated a single, homogeneous class of binding sites with K_a of 2.39 x 10^7 M^{-1} (Figure 12a). The K_a in the presence

of mouse plasma (35 mg proteins) or liver extract (100 ul of 1:8 vol:vol ethanol extraction) was 2.45×10^7 M^{-1} and 2.23×10^7 M^{-1} respectively (Figure 12b and 12c). Results indicated that there were no significant interferring substances, such as folates etc., in mouse plasma or mouse liver extract effected to the binding affinity between pyrimethamine and rat liver DHFR.

The amount of pyrimethamine in body fluids and tissues could be determined by using the standard curve. Figure 13a, 13b, 13c, 13d and 13e clearly exhibited the possible competitive of ^{14}C-pyrimethamine by cold pyrimethamine. Increasing concentration of unlabeled pyrimethamine caused the decrease in binding of ^{14}C-pyrimethamine to DHFR. The standard curve which was plotted between C_o/C_x and the concentration of cold pyrimethamine added, exhibited linearity in the range from 20 nM to 300 nM of pyrimethamine. Also the presence of mouse plasma (35 mg proteins), mouse liver extract (100 ul of 1:8 vol:vol ethanol extraction), human plasma (39 mg proteins) and human serum (49 mg proteins) gave no significant differences of the graph from the control. The slope of the standard curve with the presence of mouse liver extract seemed to be slightly lower than the value observed with the presence of mouse plasma. This might be due to the effect of ethanol to DHFR activity itself as illustrated in figure 5.

The sensitivity of this competitive binding assay for pyrimethamine was found to be 20 nM (5 ng/ml plasma or 90 ng/g liver). This was slightly higher than the value of

10 ng/ml plasma reported by DeAngelis, et al. (1975), using TLC method, but it was comparable to the HPLC method developed by Edstein (1984). The GLC method had lower limit of sensitivity of 100 ng/g tissue (Cala, et al., 1972).

The precision studies of competitive binding assay for pyrimethamine determination was given in term of percent coefficient of variation (%C.V.). The percent coefficient of variation within assay were 5.09, 4.48, 3.69, 4.48 and the value of %C.V. between assay were 5.81, 5.06, 6.07, 6.27 when having mouse plasma (35 mg proteins), mouse liver extract (100 ul of 1:8 vol:vol ethanol extraction), human plasma (39 mg proteins) and human serum (49 mg proteins) as background of samples respectively.

The accuracy of competitive binding assay for pyrimethamine evaluted from %recovery was in the range of 89.0-108.7, 84.5-99.4, 99.3-122.2 and 80.2-100.5 when having mouse plasma (35 mg proteins), mouse liver extract (100 ul of 1:8 vol:vol ethanol extraction), human plasma (39 mg proteins) and human serum (49 mg proteins) respectively. It is worth noting that the calculated %recovery of pyrimethamine with the presence of human plasma became close to 100% when the value of pyrimethamine detected were at lower range but it was more fluctuated for the higher level of pyrimethamine. This might be due to the wide range of the standard variation observed in standard curve when higher amount of pyrimethamine was added or from some interfering substances present in the background samples.

The specificity of binding between pyrimethamine and DHFR was also studied and the results are shown in table 4. Folate analogues such as folic acid, tetrahydrofolate, 5-methyl-tetrahydrofolate and leucovorin (5-formyl-tetrahydrofolate) were used as the model interfering substances since small amount of these folates has been reported to be present in mammalian plasma. The concentration of the folates which inhibited 50% binding capacity of ^{14}C-pyrimethamine to DHFR was used as index. It can be seen from table 4 that all the folate analogue tested exhibited the inhibition of ^{14}C-pyrimethamine binding less than cold pyrimethamine itself under the same conditions. The concentration of leucovorin used was about 10^4 times higher than the value obtained from pyrimethamine, whereas the concentration of folic acid, tetrahydrofolate and 5-methyl-tetrahydrofolate used were about 10^3 times higher. Sulphanilamide failed to inhibit ^{14}C-pyrimethamine binding at concentration up to 1×10^{-3} M. The normal physiological value of folate in human plasma was reported to be about 1.37×10^{-7} M for folic acid (Orter and Neuhceus, 1982) and for 5-methyl-tetrahydrofolate which is the major form of circulating folate is around 3×10^{-8} M (Johns and Bertino, 1965). The values of 50% inhibition of folic acid and 5-methyl-tetrahydrofolate were obviously far higher than the physiological levels.

In order to determine the level of pyrimethamine in body fluids and tissues, ti had to begin with the extraction of drug by the organic solvent. Plasma and urine containing pyrimethamine were made alkaline (pH > 12) by adding

sodium hydroxide then extracted with ethylene dichloride (Schmidt, et al., 1953; Jones and King, 1968; Simmon and DeAngelis, 1973 and Edstein, 1984). Other organic solvent used for the extraction of pyrimethamine from plasma were 1,2-dichloroethane (DeAngelis, et al., 1975 and Jones and Ovenell, 1979), toluene (Jones, et al., 1981) and n-butyl-chloride-dichloromethane (Timm and Weidekamm, 1981). For tissue samples, DeAngelis, et al. (1975) extracted pyrimethamine by homogenized tissue samples with lactic acid. After centrifuged, the supernatant was neutralized with sodium hydroxide and extracted with 1,2-dichloroethane. Ploydanai (1982) used 1% lactic acid plus 10% trichloroacetic acid to extract pyrimethamine from human red blood cells. Nuchadomrong (1985) also reported that 95% ethanol could be used for the extraction of pyrimethamine from mice red blood cells.

The competitive binding assay method which had been developed in this research project can be used to determine the level of pyrimethamine in intact crude serum and plasma of mice and human without any previous extraction. However, 95% ethanol was needed to use as the solvent for the extraction of pyrimethamine from liver and red blood cells of mice. Nuchadomrong (1985) reported that %recovery of the extraction of pyrimethamine from red blood cells of mice was almost 100% when 95% ethanol was used.

The optimum amount of 95% ethanol to extract pyrimethamine from liver homogenate and red blood cells had been determined as illustrated in figure 14a and 14b.

Having 12.5 nM ^{14}C-pyrimethamine in the liver homogenate or red blood cells, the optimum ratio of liver homogenate or red blood cells to extracting 95% ethanol used was found to be 1:5 (vol:vol) or more for maximum recovery of pyrimethamine (90-95%). If the extractable pyrimethamine in liver homogenate or red blood cells was increased to 12.5 nM ^{14}C-pyrimethamine plus 0.1 mM pyrimethamine, the optimum ratio of liver homogenate or red blood cells to extracting 95% ethanol was increased to 1:8 (vol:vol) for both sources with about 95% of pyrimethamine recovered. Thus, the ratio of 1:8 (vol:vol) was selected for the extraction of pyrimethamine from mouse liver and red blood cells since it was the optimum ratio which gave highest recovery.

In the second portion of this thesis, the developed competitive binding assay was used to determine the level of pyrimethamine in plasma, liver and red blood cells of uninfected mice and mice infected with *P. chabaudi* clone AS and AS(Pr_1). *P. chabaudi* clone AS, modeling as drug-sensitive malaria parasites, was susceptible to pyrimethamine 15 mg/kg body wt, whereas *P. chabaudi* clone AS(Pr_1), modeling as drug-resistant malaria parasite, was susceptible to pyrimethamine 30 mg/kg body wt. The uninfected and infected mice were treated with single dose of pyrimethamine by oral (5 mg/kg body wt) or intraperitoneal (5 and 30 mg/kg body wt) administration on day 3 after 10^8 parasitized erythrocytes inoculation. The %parasitemia, pattern of parasitic clearance and death, including rate and level of pyrimethamine uptake, time of drug retainment and elimination

rate of pyrimethamine were compared in the previously mentioned system. Hopefully, the results gained would help to understand and adding some more informations of the mechanism and biochemical aspect of the infection process in pyrimethamine resistant plasmodium parasite.

The fundamental pattern of changes in parasitemia for mice infected with *P. chabaudi* AS or *P. chabaudi* AS(Pr_1) without pyrimethamine treatment was firstly checked. On the first day after 10^8 of parasitized cells had been inoculated, percent parasitemia was fairly low (about 5 %) (Figure 15a and 15b). This was followed by invasion of plasmodium into the liver parenchymal cells and developed into the maturation stage. The merozoites then came out into the blood stream and multiplied. The number of parasitized cells increased in the logarithmic pattern. The untreated mice died when percent parasitemia increased to 80-90 percents (on day 4-5 after the infection).

When *P. chabaudi* infected mice were treated with pyrimethamine on day 3 after inoculation; the parasitemia of mice infected with both clones of *P. chabaudi* (AS and AS(Pr_1)) was decreased due to the inhibition of pyrimethamine. This schizonticide drug was reported to act on the schizont stage of parasitized cell growth which then decreased the amount of merozoites to reinvade the new erythrocytes, so the number of parasitemia cells were reduced. However, in all groups of pyrimethamine treated infected mice, the %parasitemia became higher again after reduction for a certain time. The recrudescent parasitemia peak was

followed by gradually diminished in number of parasite down to the base line within a few days. Continuing analysis for the presence of parasitized cells in the blood circulation system indicated absolute clearance of the parasite up to 3 months.

When mice were treated with pyrimethamine orally (5 mg/kg body wt), the appearance of the recrudescent parasitemia peak in mice infected with *P. chabaudi* AS seemed to occur at a longer period of time after drug treatment in comparison with the group of mice infected with *P. chabaudi* AS(Pr_1) (Figure 16a and 16b). This probably due to the higher uptake of pyrimethamine in mice infected with *P. chabaudi* AS than mice infected with *P. chabaudi* AS(Pr_1), so the amount of parasite killed should be greater and leading to a longer time of retainment.

Ploydanai (1982) studied the uptake of pyrimethamine into *P. falciparum* infected red blood cells and reported that the uptake of drug into the cells infected with sensitive isolate of plasmodium was significantly higher than the pyrimethamine resistant infected and normal red blood cells respectively.

In vitro studies of pyrimethamine uptake into red blood cells of mice infected with *P. chabaudi* exhibited the corresponding results that the uptake of pyrimethamine in *P. chabaudi* AS (pyrimethamine sensitive) infected red blood cells was higher than *P. chabaudi* AS(Pr_1) (pyrimethamine resistant) infected and normal red blood cells, respectively (Nuchadomrong, 1985).

The pattern of changes in parasitemia of *P. chabaudi* AS and AS(Pr_1) infected mice after receiving pyrimethamine (5 mg/kg body wt) intraperitoneally were similar to that observed for the oral administration, expect that the appearance of the recrudescent parasitemia peak occurred at a shorter period of time. The recrudescent parasitemia peaks observed were obviously higher than mice treated with pyrimethamine orally (Figure 20a and 20b). These results convincingly supported that oral administration of pyrimethamine (5 mg/kg body wt) was probably highly effective in the malarial therapy of mice than the intraperitoneal administration.

So far, there are only few reports on the effective routes of drug administration in preventing or treating malarial infections. Ferraroni and Speer (1982) reported that oral administration of Fansidar (1.0 mg pyrimethamine and 20.0 mg sulfadoxine/kg body wt) was more effective in reducing or preventing parasitemia than intramuscular injection.

The remarkably differences in the time of the recrudescent parasitemia peak of infected mice treated with 30 mg/kg body wt of pyrimethamine intraperitoneally from the group of mice treated with 5 mg/kg body wt of pyrimethamine either orally or intraperitoneally had been demonstrated in figure 22a and 22b. These might be due to the higher amount of pyrimethamine uptake into plasma which caused the longer period of time for pyrimethamine to retain in the parasites and relative organs of the host and

suppressed the parasites to sub-lethal levels. The above data leading to the assumption that the period of time for the recrudescent parasitemia occurred was influenced by the dosage of pyrimethamine and the route of drug administration.

The measurement of pyrimethamine level in plasma, liver and red blood cells of *P. chabaudi* infected and uninfected mice when they were treated with single dose of pyrimethamine on day 3 after parasite inoculation was performed by using the competitive binding assay method. The values obtained were calculated from the standard curve using C_o/C_x within the range of 20-300 nM.

The results when mice were treated with single dose of pyrimethamine orally (5 mg/kg body wt) exhibited a clearly different patterns of pyrimethamine turnover in uninfected and infected mice (Figure 17a, 17b and 17c). They were less accumulation of pyrimethamine in uninfected mice incomparison with the infected ones. The levels of pyrimethamine detected in plasma of mice infected with *P. chabaudi* AS were slightly higher than that of mice infected with *P. chabaudi* AS(Pr_1) and uninfected mice, respectively. The elimination rate of pyrimethamine from plasma of uninfected mice (estimated elimination half-life ~4-5 hr) was more rapidly than the group of mice infected with both clones of *P. chabaudi* (estimated elimination half-life ~9-10 hr). No significant differences in rate of pyrimethamine uptake in all three groups of mice studied were detected. However, the lasting time of pyrimethamine retainment was illustrated to be

highest in mice infected with *P. chabaudi* AS (> 48 hr) and shortest in uninfected mice (18 hr). The similar pattern of pyrimethamine turnover was also found when assays were performed in mouse liver and red blood cells (Figure 18a, 18b, 18c and Figure 19a, 19b, 19c). The drug seemed to have accumulated at the higher level (~28 nmole pyrimethamine/g liver and estimated elimination half-life ~10 hr) in the liver of mice infected with *P. chabaudi* clone AS than clone AS(Pr_1) (~18 nmole pyrimethamine/g liver and estimated elimination half-life ~ 8 hr). The lowest level of pyrimethamine accumulation was observed in the liver of uninfected mice (~11 nmole pyrimethamine/g liver and estimated elimination half-life ~ 5 hr). Only slight level of pyrimethamine could be detected in red blood cells of uninfected mice (0.03 nmole/10^{12} rbc) incomparison with the value of 0.29 and 0.23 nmole/10^{12} rbe detected in red blood cells of mice infected with *P. chabaudi* AS and *P. chabaudi* AS(Pr_1) respectively. The results observed were clearly supported from the data obtained by Nuchadomrong (1985) for *in vitro* studies of pyrimethamine uptake in *P. chabaudi* AS and AS(Pr_1) infected red blood cells.

Intraperitoneal injection of pyrimethamine (5 mg/kg body wt) into *P. chabaudi* infected and uninfected mice exhibited a resemble pattern of pyrimethamine turnover in plasma as was found in mice treated orally with the same dosage of pyrimethamine (Figure 21a, 21b and 21c). The highest levels of pyrimethamine detected were at 0.5 hr after drug injection. This indicated that pyrimethamine

was uptaken into plasma at higher rate for the intraperitoneal than the oral treatment.

For mice treated with higher dose of pyrimethamine (30 mg/kg body wt) intraperitoneally, the levels of pyrimethamine detected in plasma were remarkably higher than mice received pyrimethamine (5 mg/kg body wt) either orally or intraperitoneally. The significant levels of pyrimethamine (~0.5, 0.2 and 0.1 nmole of pyrimethamine/ml plasma in uninfected mice, mice infected with *P. chabaudi* AS and mice infected with *P. chabaudi* AS(Pr_1) respectively) could still be detected at 48 hr after drug administration (Figure 23a, 23b and 23c). Hereagain, the rate of pyrimethamine uptake into plasma was faster than the oral treatment. This obviously indicated that the higher dose of drug was still be able to uptake into plasma directly.

It has long been reported that when drug is given intraperitoneally, its absorption is almost complete but the rate at which the drug enters the circulation depended upon local blood flow and the state of ionization of the drug. Whereas, when the drug is given orally, both the amount absorbed and the rate of absorption are determined by many factors, especially the physical nature of the dosage form, the presence of food in the stomach, the composition of the gastrointestinal motility, the mesenteric blood flow and the concurrent oral administration of other drugs (Mayer, et al., 1980).

It was suggested by Wartak (1983) that the whole dose of drug administration never enter the systematic

circulation except that drug injected intravenously. A major fact that can reduce the dose of drug is extension metabolism in the liver during the first passage to that organ. Benet (1978) pointed out that orally absorbed drug must enter liver via the portal vein before reaching the systematic circulation. The average maximum percentage recovery of pyrimethamine in blood circulation, liver and red blood cells of mice received pyrimethamine 5 mg/kg body wt orally was not exceeded 15%. The higher level of pyrimethamine uptake into the uninfected and *P. chabaudi* infected mice can be demonstrated in the plasma of mice injected with pyrimethamine 30 mg/kg body wt.

After pyrimethamine administration, either 5 or 30 mg/kg body wt, the parasitemia dropped. This might be due to the direct action of pyrimethamine on the malarial parasites. Thereafter, the parasitemia rose again and this relapse parasitemia could be arose from the latent forms of parasite of various sources, such as liver etc., which were subsequently released into the blood stream. From the results obtained in figure 16, 20 and 22, it was obviously demonstrated that the recrudescent peak of parasite dissappeared within a few days after the occurrence, although, the level of pyrimethamine in plasma, liver and red blood cells were very low (Figure 17, 18, 19, 21 and 23). The declining of the percent parasitemia in recrudescent peak was not expected to be the direct action of pyrimethamine itself but possibly from other mechanisms, such as the host immune response induced by the parasite.

In human, the elimination half-life of pyrimethamine was 95.5 ± 30.6 hr (Weidekamm, et al., 1982), 84.3 hr (Cavallito, et al., 1978), 77.4 hr (Jones and Ovenell,1979), 118.3 hr (Yimm and Weidekamm, 1982).

It has been reported that malarial parasites caused changes in almost every component of the immune system (Playfair, 1982). Primary infection of *P. falciparum* gave rise to the response of IgG, IgA and IgM changes within the first week (Collins, et al., 1971). Human infected with *P. falciparum* or *P. vivax* will lead to the remarkably increment of α-globulins in 10 days and maximum synthesis of γ-globulins was demonstrated at day 20 (Collins, et al., 1971).

McLean, et al. (1982) had examined the relationship between acquired immunity of the host and recrudescences using *P. chabaudi* in the mouse. Mice were infected with 10^6 *P. chabaudi* parasitized erythrocytes. The infection showed an acute parasitemia which became subpatent after about 2 weeks. A period (7-10 days) of subpatency followed before a short lasting patent recrudescence appeared. The mean antibody levels in sera collected during the course of infection rose during the first patent parasitemia and then declined slightly in the subpatent period between the first parasitemia and the recrudescence. During the recrudescence the titre again rose and declined slowly after the declining of the recrudescence parasitemia.

In conclusion, the data obtained from this research project suggested that the effective dose of pyrimethamine for the therapeutic action of mice infected with *P. chabaudi* clone AS and AS(Pr_1) as high as 20-40% parasitemia was 5 mg/kg body wt with single treatment both oral and intraperitoneal administration. The mechanism of pyrimethamine therapeutic action in mice infected with *P. chabaudi* might be due to the retainment of parasite at the level of sublethal dose for a certain period of time and then the function of immune response to the infection of infected plasmodium itself was involved directly to the clearance of parasites from the whole system.