Journal of Chromatography, 487 (1989) 51–59
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4497

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THYMIDYLATE SYNTHASE FROM THE HUMAN MALARIA PARASITE, *PLASMODIUM FALCIPARUM*

JERAPAN KRUNGKRAI*,*

Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Rama 4 Road, Bangkok 10500 (Thailand)

YONGYUTH YUTHAVONG

Department of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400 (Thailand)

and

H. KYLE WEBSTER

Department of Immunology and Biochemistry, Armed Forces Research Institute of Medical Sciences, Rajavithi Road, Bangkok 10400 (Thailand)

(First received April 11th, 1988; revised manuscript received September 12th, 1988)

SUMMARY

A rapid and highly sensitive high-performance liquid chromatographic assay for thymidylate synthase activity is described. The assay is based on the separation of the substrate, deoxyuridylate (dUMP), and its product, deoxythymidylate (dTMP), on a LiChrosorb RP-8 reversed-phase column with 44 m*M* triethylammonium phosphate (pH 7.0) as mobile phase and a flow-rate of 1.0 ml/min. In addition, using a μBondapak C_{18} reversed-phase column with 10 m*M* potassium phosphate (pH 4.0) and a gradient of 0–28% methanol, dUMP, dTMP and deoxythymidine (dTdR) are well separated within 30 min. The latter system is also applied to assay thymidine kinase activity with dTdR and dTMP as substrate and product, respectively. This method is sensitive enough to measure dTMP at concentrations as low as 25 pmol, and it was used to show that crude extracts of the human malaria parasite *Plasmodium falciparum* contain thymidylate synthase but not thymidine kinase activity.

INTRODUCTION

Thymidylate synthase (methylenetetrahydrofolate; 2′-deoxyuridine 5′-monophosphate C-methyltransferase; EC 2.1.1.45) catalyses the reaction:

*Address for correspondence: Laboratory of Medical Biochemistry, The Rockefeller University, 1230 York Avenue, New York, NY 10021, U.S.A.

0378-4347/89/$03.50 © 1989 Elsevier Science Publishers B.V.

deoxyuridylate (dUMP) + 5,10-methylene-H_4PteGlu(MTHF) ⟶ deoxythymidylate (dTMP) + H_2PteGlu (dihydrofolate)

Thymidylate synthase is a target enzyme for chemotherapy of cancer and infectious diseases [1,2] since it provides dTMP for DNA synthesis. The usual method for the enzyme assay is based on radiometric measurement of ^3H_2O released from reductive methylation of [5-^{3}H] dUMP by MTHF to give dTMP and H_2PteGlu as products [3,4]. In addition, spectroscopic [5,6] and acrylamide gel electrophoretic [6,7] methods have been used. Recently, 5′-pyrimidine and 5′-deoxypyrimidine mononucleotides were separated by reversed-phase high-performance liquid chromatography (HPLC), which allows quantification of the enzymes pyrimidine 5′-nucleotidase and deoxypyrimidine 5′-nucleotidase in human red blood cells (RBCs) [8].

This report describes the use of two reversed-phase HPLC techniques to quantify the activity of thymidylate synthase in the human malaria parasite, *Plasmodium falciparum*, by the separation of the substrate dUMP from the product dTMP with a highly sensitive level of detection. In addition, the activity of thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) in the parasite was also examined by measuring the production of dTMP separated from the substrate deoxythymidine (dTdR) by reversed-phase HPLC.

EXPERIMENTAL

Materials

Triethylamine, saponin, tris(hydroxymethyl)aminomethane, dUMP, dTMP, dTdR, 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) and tetrahydrofolic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Potassium dihydrogenphosphate, methanol, 2-propanol and 85% phosphoric acid were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The protein assay kit was obtained from Bio-Rad (Richmond, CA, U.S.A.). Ficoll-Paque® and Sephadex G-25 were obtained from Pharmacia (Piscataway, NJ, U.S.A.). All other chemicals were purchased from Sigma.

HPLC apparatus and chromatographic conditions

The HPLC system was a Spectra-Physics (Santa Clara, CA, U.S.A.) Model SP8100 liquid chromatograph equipped with a Valco Model C6U injector (50-μl loop), a Model SP8440 variable-wavelength detector and a Model SP4270 computing integrator.

dUMP and dTMP were separated using a LiChrosorb RP-8, 5-μm column (250 mm × 4.6 mm I.D.) (Spectra-Physics) at room temperature (25 ± 2°C) and a flow-rate of 1.0 ml/min. The mobile phase was triethylammonium phosphate buffer (pH 7.0) with the following composition: 2-propanol–85% phosphoric acid–triethylamine–triply distilled water (3:3:10:984, v/v) as previously described for a GTP cyclohydrolase assay [9]. The UV absorbance of the effluent was monitored at 254 nm. The two pyrimidine nucleotides were also separated on a μBondapak C_{18} column (300 mm × 3.9 mm I.D., particle size 10 μm) (Waters Assoc.,

Milford, MA, U.S.A.) in conjunction with a Corasil μBondapak C_{18} (27–40 μm) guard column. The eluting solvent (solvent A) was 0.01 *M* potassium phosphate buffer (pH 4.0) at a flow-rate of 1.0 ml/min. A programmed gradient elution of 70% methanol (solvent B) to 60% solvent A and 40% solvent B at 30 min was applied after 12 min of 100% solvent A. The mobile phase condition was modified from Cook et al. [8].

The separation of the two pyrimidine nucleotides was also achieved using strong anion-exchange HPLC on a Partisil 10 SAX column (250 mm × 4.6 mm I.D., particle size 10 μm) (Whatman, Clifton, NJ, U.S.A.) with gradients of 0.01 *M* potassium dihydrogenphosphate (pH 3.4) and 0.8 *M* potassium dihydrogenphosphate (pH 4.3) [10]. The effluent was monitored at 254 nm, and retention times and peak areas were computed by an SP 4270 integrator. The compounds were quantified by area integration using an external standard method.

Enzyme preparations

The human malaria parasite, *P. falciparum*, was maintained in culture by the method of Trager and Jensen [11]. Intact parasites were obtained by saponin lysis of the parasitized RBCs. The parasite extract (freeze–thaw lysate) was used directly for the enzyme assay. Protein concentration was determined by the method of Bradford [12], using bovine serum albumin as standard.

Human lymphocytes were isolated from whole blood using Ficoll-Paque density gradient medium. Enzyme was prepared from recovered lymphocytes by freeze–thaw extraction. In some experiments, the enzyme extracts were applied to a Sephadex G-25 column (2-ml bed volume) to separate the enzyme from low-molecular-mass contaminants.

Enzyme assay

Thymidylate synthase was assayed by detecting the product, dTMP, separated from the substrate, dUMP, using the described HPLC methods. In the enzyme assay of 250 μl total volume, the enzyme was preincubated at 37°C for 5 min in a reaction mixture containing 50 m*M* Tris–HCl buffer (pH 7.8), 50 m*M* 2-mercaptoethanol, 5 m*M* formaldehyde and 1 m*M* tetrahydrofolate. The reaction was initiated by adding 25 μl of the dUMP solution to give a final concentration of 0.1 m*M* and then allowed to proceed at 37°C for 30 min. The incubation conditions for assay of thymidylate synthase were modified from the previous reports [3,13]. After incubation, 250 μl of ice-cold 1 *M* perchloric acid were added to stop the reaction, and the reaction mixture was then placed in an ice-bath for 30 min. The supernatant fluid, after centrifugation in an Eppendorf microfuge for 5 min, was neutralized with 0.1 volume of 10 *M* potassium hydroxide containing 1 *M* potassium dihydrogenphosphate [14]. The mixture was then centrifuged for 5 min. The supernatant was kept at −20°C and recentrifuged before injection onto the HPLC column.

For the thymidine kinase assay, the enzyme activity was measured in 250 μl of reaction mixture containing 50 m*M* Tris–HCl (pH 7.8), 50 m*M* 2-mercaptoethanol, 5 m*M* ATP and 5 m*M* magnesium chloride. The reaction was started by adding 0.1 m*M* dTdR (final concentration) and then incubated at 37°C for 30

min. The incubation conditions were slightly modified from those of Her and Momparler [15]. The reaction was terminated with 250 μl of ice-cold 1 M perchloric acid, and the subsequent steps were performed as for thymidylate synthase.

RESULTS

The dUMP and dTMP could not be resolved on the strong anion-exchange column (Partisil 10 SAX column) with gradients of 0.01 M potassium dihydrogenphosphate (pH 3.4) and 0.8 M potassium dihydrogenphosphate. The separation of dUMP and dTMP was, however, achieved within 20 min on an ion-paired LiChrosorb RP-8 column with 44 mM triethylammonium phosphate (pH 7.0) containing 0.15% 2-propanol as the mobile phase (Fig. 1A). The retention times (t_R) of dUMP and dTMP were highly reproducible [t_R for dTMP = 14.85 ± 0.09 min; n=9, coefficient of variation (C.V.) = 1.0%]. It was found that dUMP and dTMP were well resolved on a reversed-phase μBondapak C_{18} column with 0.01 M potassium phosphate (pH 4.0) as mobile phase and a flow-rate of 1.0 ml/min (Fig. 1B). The values of t_R for dUMP and dTMP were 6.18±0.16 min (C.V.=2.0%, n=8) and 9.36±0.24 min (C.V.=3.0%, n=8). To separate dTdR, a gradient of methanol (up to 28%) was applied on the μBondapak C_{18} column after dUMP and dTMP had been separated (at 12 min); dTdR was eluted at 23.88±0.23 min (C.V.=2.9%, n=8) as shown in Fig. 1C.

The chromatographic pattern of the thymidylate synthase reaction with a crude extract of *P. falciparum* as the enzyme source is shown in Fig. 2. The experimental incubation with 250 μl (0.48 mg of protein) of the enzyme solution from a crude

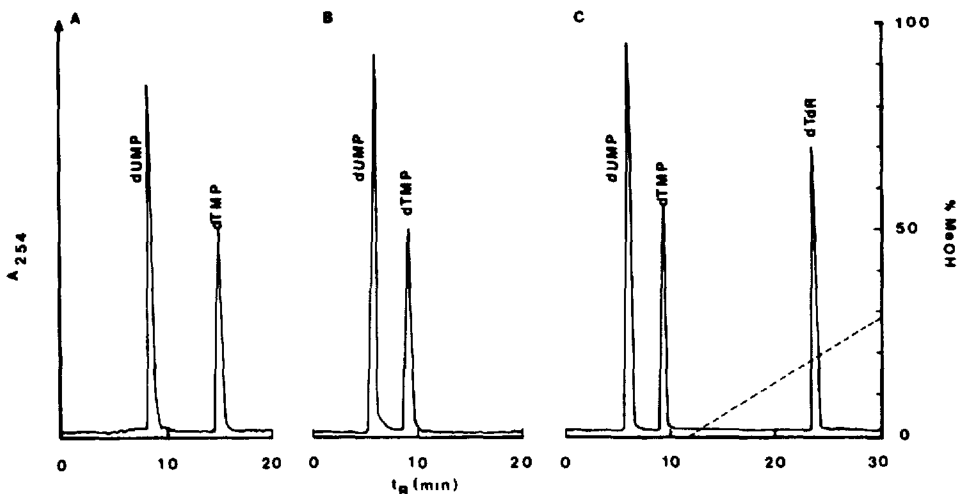

Fig. 1. Chromatographic separation of dUMP, dTMP and dTdR. The concentration of each compound injected was 25 nmol/ml in a sample volume of 50 μl. (A) LiChrosorb RP-8 column (5 μm) and 0.044 M triethylammonium phosphate buffer (pH 7.0) with a flow-rate of 1.0 ml/min (B) μBondapak C_{18} column (10 μm) with Corasil μBondapak C_{18} (27–40 μm) as guard column and 0.01 M potassium phosphate (pH 4.0) at a flow-rate of 1.0 ml/min. (C) Conditions same as B, except that after 12 min a gradient from 0 to 40% of 70% methanol over 30 min was applied.

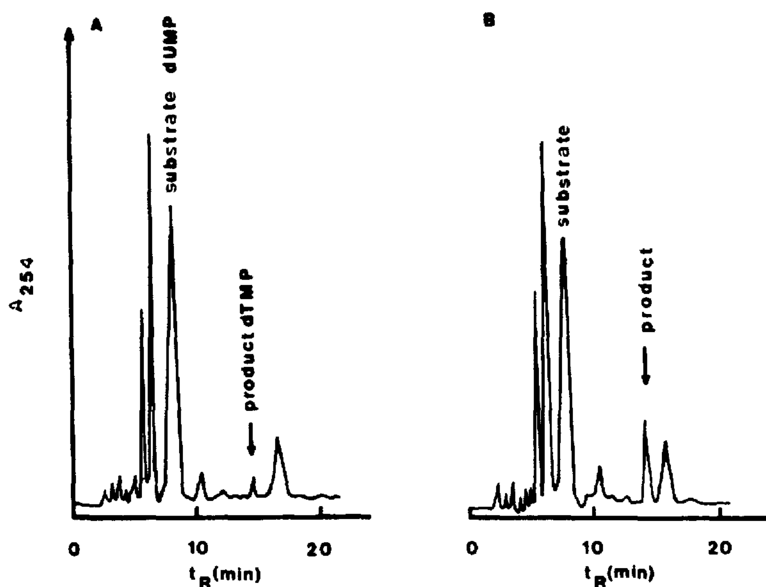

Fig. 2. Chromatograms of a 50-μl aliquot of the enzyme thymidylate synthase incubation mixture from an extract of *P. falciparum*. Conditions as in Fig. 1A. (A) Incubation for 0 min, (B) incubation for 30 min.

TABLE I

EFFECT OF INCUBATION CONDITIONS ON HPLC ASSAY OF THYMIDYLATE SYNTHASE

The conditions are described in Experimental. The incubation mixture in experiments 1, 2 and 3 contained 480, 674 and 510 μg of enzyme, respectively. The reactions were stopped with perchloric acid–potassium hydroxide–potassium dihydrogenphosphate in a final volume of 540 μl. Only 50 μl of the extracts were injected.

Condition	dTMP detection				
	Peak area experiments 1-3 (mean ± S.D.)	Concentration (pmol)			
		Exp. 1	Exp. 2	Exp. 3	Mean ± S.D.
0 min incubation	27 268±8350	93.75	154.82	107.11	118.56±32.10
30 min incubation	83 368±9482	333.63	403.44	350.32	362.46±36.45
30 min (boiled enzyme)	28 131±5421	105.33	145.57	116.01	122.30±20.84
30 min (0.1 m*M* FdUMP)	25 723±8073	87.84	146.90	100.79	111 84±31.02

extract of *P. falciparum* incubated at 37°C for 30 min (Fig. 2B) shows the formation of dTMP, even when the sample injection volume was only 50 μl out of the total volume of 540 μl after extraction with perchloric acid–potassium hydroxide–potassium dihydrogenphosphate, amounting to 44 μg of protein of the crude extract. There was a significant amount of endogenous dTMP in the enzyme incubation mixture (see Fig. 2A). The same amount of endogenous dTMP was shown also for the control incubations (Table I), in which the enzyme was

boiled before being added to the reaction or incubated in the presence of 0.1 mM FdUMP, a potent inhibitor of thymidylate synthase [13].

The rate of dTMP formation using the enzyme from crude extracts of *P. falciparum* proceeded linearly up to 30 min at 37°C. The 30-min incubation was used for determination of the enzyme activity in crude extracts of the parasites. The thymidylate synthase activity as a function of enzyme concentration is shown in Fig. 3. The formation of dTMP was linear with the enzyme concentration between 80 and 720 μg of protein of the crude extract.

As little as 25 pmol of dTMP formed from the enzyme reaction could be detected by UV absorption at 254 nm. The A_{254}/A_{280} ratio was constant at 1.77. The UV detector response of dTMP was linear from 25 pmol to 5 nmol. The recoveries of dUMP and dTMP (0.5 and 1.0 nmol) added to the enzyme reaction mixture without the substrate were 99.4 and 103.6% respectively. With potassium hydroxide–potassium dihydrogenphosphate as the neutralizing agent in perchloric acid extracts, dUMP and dTMP peaks showed no degradation compared with the distorted peaks of dUMP and dTMP obtained from extraction with only perchloric acid and potassium hydroxide. It was also shown that the t_R of the distorted peaks were changed with the latter treatment, which results in an acidic pH (< 4).

A reversed-phase HPLC separation of dUMP and dTMP (Fig. 1B) was also

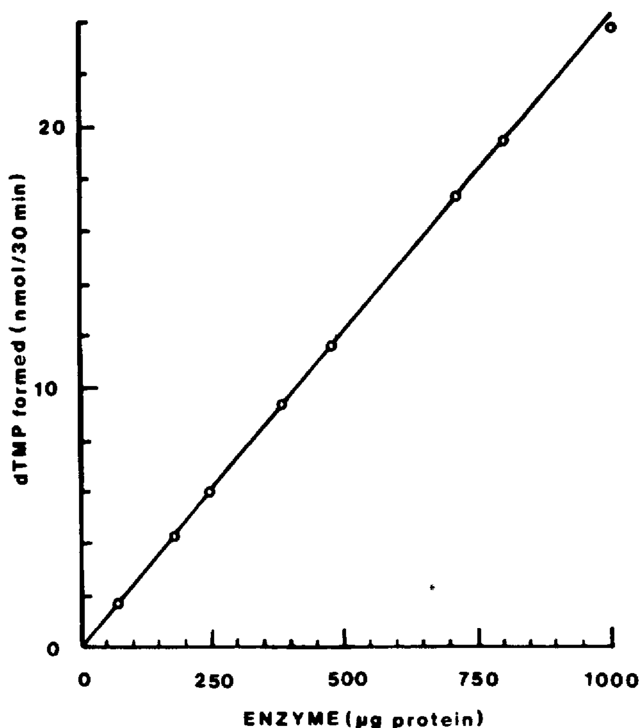

Fig. 3 Thymidylate synthase activity in a crude extract of *P. falciparum* as a function of enzyme concentration. For conditions, see Experimental.

TABLE II

THYMIDYLATE SYNTHASE AND THYMIDINE KINASE ACTIVITIES IN *P. FALCIPARUM* AND HUMAN LYMPHOCYTES

The procedure was as described in Experimental, except that thymidine kinase assay was carried out at 37°C for 60 min.

Source	Enzyme activity*	
	Thymidylate synthase	Thymidine kinase
P. falciparum (K_1 isolate)**	0.886±0.141 (n=8)	Not detectable
P falciparum (G_{112} isolate)**	1.252±0.244 (n=5)	Not detectable
Human lymphocytes	0.165±0.038 (n=4)	0.647±0.118 (n=4)

*The enzyme activity was expressed in nmol of dTMP formed per min per mg protein, and the results represent the mean ± S.D.
*******P. falciparum* was from cultures with 8–10% parasitemia, with trophozoites and schizonts as predominant forms. The K_1 and G_{112} isolates were pyrimethamine-resistant and -sensitive parasites, respectively.

used for detection of the enzymic reaction in crude extract of *P. falciparum.* The chromatograms of the enzyme assay following incubation for 0 min and 30 min are not much different from Fig. 2. However, a methanol gradient was applied following the appearance of dUMP and dTMP peaks, in order to elute the other substrate (MTHF), and the column required at least 14 min for equilibration prior to the next analysis.

Another enzyme, thymidine kinase, was also measured using the HPLC method described in Fig. 1C. Crude extracts of *P. falciparum* showed no dTMP formation after 30-min incubation, whereas the human lymphocyte lysate contained a detectable enzyme activity. The activities of the enzymes thymidylate synthase and thymidine kinase in crude extracts of *P. falciparum* (K_1 and G_{112} isolates) and human lymphocytes are given in Table II.

DISCUSSION

Published methods for the assay of thymidylate synthase are based on the separation of ^3H_2O from [5-^{3}H]dUMP after incubation of the enzyme with MTHF on a charcoal column [3,4]. The estimation of ^3H_2O eluted from the charcoal column is subject to considerable variation and substantial background [3,4,13]. Although these methods are suitable when there are large sample numbers and volumes, they have drawbacks when high sensitivity is needed.

We have applied HPLC methods for measuring thymidylate synthase activity in crude extracts of *P. falciparum* and human lymphocytes. Two reversed-phase HPLC systems, an ion-pair system with isocratic elution on a LiChrosorb RP-8 column and a gradient elution system on a μBondapak C_{18} column, were developed for separation of the substrate dUMP from the product dTMP. The ion-pair HPLC method was the more practical, in that the running time was shorter (20 min) and no equilibration of the column prior the next injection was neces-

sary. Both methods gave sharp peaks for dUMP and dTMP. Nevertheless, dUMP and dTMP were not resolved in the gradient system on the Partisil 10 SAX column, as described earlier for purine nucleotides [10].

The HPLC assay methods for thymidylate synthase activity reported here have many advantages. They are simple and highly sensitive, capable of detecting 25 pmol of dTMP formed in the enzymic reaction. Since the recoveries of both dUMP and dTMP were very high (99.4 and 103.6%) following extraction with perchloric acid–potassium hydroxide–potassium dihydrogenphosphate, the amount of the enzyme from a crude extract of *P. falciparum* could be reduced to as low as 9 μg of protein in 50 μl of reaction mixture for the limitation of UV detection (25 pmol). The amount of protein from crude extracts of *E. coli* for detecting thymidylate synthase activity by the radiometric assay was ca. 5 μg [4]. These techniques were, therefore, suitable for microassay of the enzyme. It was noted that, at such high sensitivity, a significant amount of dTMP in the control incubation was detected, as shown in Table I. Without the extract in the reaction medium (enzyme blank), there was no dTMP detected, indicating that the crude extract contained some endogenous dTMP.

The extraction procedure of the enzyme incubation mixture by perchloric acid and neutralization with potassium hydroxide–potassium dihydrogenphosphate resulted in sharp peaks for both dUMP and dTMP. The deproteinized reaction mixture could be directly analysed by simultaneous HPLC. The methods also allowed simultaneous determination of dTMP and dUMP, enabling both the rates of substrate utilization and product formation to be obtained for verification of reaction specificity. However, MTHF, the other substrate, and H_2PteGlu, the other product, were not separated in these systems and were eluted later during chromatography. The two folates in the reaction could be separated in another HPLC system [16]. In addition, the HPLC thymidylate synthase assay was less expensive than the radiometric assays in which [5-^{3}H]dUMP was required.

Thymidine kinase activity was also assayed using the HPLC method described here. The malaria parasite lacked thymidine kinase but contained high activity of thymidylate synthase, confirming the evidence of Reyes et al. [17] that *P. falciparum* is unable to utilize exogenous pyrimidines and must synthesize them de novo.

In summary, the HPLC methods for the assay of both thymidylate synthase and thymidine kinase reported here are simple, sensitive and relatively inexpensive. They should be useful for the assay of these enzymes in other biological samples and also for testing the effect of drug on enzyme activity.

ACKNOWLEDGEMENTS

This work was supported in part by the Rockefeller Foundation and by a grant from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, as well as the Walter Reed Army Institute of Research, U.S. Army Research and Development Command.

REFERENCES

1 P.V. Danenberg, Biochim. Biophys. Acta, 473 (1977) 73–92.

2 D.V. Santi, and P.V. Danenberg, In R.L. Blakley and S.J. Benkovic (Editors) Folate and Pterins, John Wiley and Sons, New York, Vol. 1, 1984, pp. 345-398.
3 K. Al Chalabi and W.E. Gutteridge, Biochim. Biophys. Acta, 481 (1977) 71-79.
4 H.O. Kammen, Anal. Biochem., 17 (1966) 553-556.
5 R.L. Blakley and B.M. McDougall, J. Biol. Chem., 239 (1962) 812-818.
6 H. Donato, J L. Aull, J.A. Lyon, J.W. Reinsch and R.B. Dunlap, J. Biol. Chem., 251 (1976) 1303-1310.
7 J. Zorzopulos, B. Raj and L.M. Kozloff, Anal. Biochem., 135 (1983) 128-133.
8 L. Cook, M. Schafer-Mitchell, C. Angle and S. Stohs, J. Chromatogr., 339 (1985) 293-301.
9 J. Krungkrai, Y. Yuthavong and H.K. Webster, Mol. Biochem. Parasitol., 17 (1985) 265-276.
10 H.K. Webster and J.M. Whaun, J. Chromatogr., 209 (1981) 283-292.
11 W. Trager and J.B. Jensen, Science, 193 (1976) 673-675.
12 M.M. Bradford, Anal. Biochem., 72 (1976) 248-254.
13 S. Pattanakitsakul and P. Ruenwongsa, Int. J. Parasitol., 14 (1984) 513-520.
14 C. Garrett and D.V. Santi, Anal Biochem., 99 (1979) 268-273.
15 M.O. Her and R.L. Momparler, J. Biol. Chem., 246 (1971) 6152-6158.
16 J. Krungkrai, H.K. Webster and Y. Yuthavong, unpublished results.
17 P. Reyes, P.K. Rathod, D.J. Sanchez, J.E.K. Mrema, K.H. Rieckman and H.-G. Heidrich, Mol. Biochem. Parasitol., 5 (1982) 275-290.