

*Full Length Research Paper*

# High performance liquid chromatographic determination of proguanil after derivatisation with sodium benzoxazole-2-sulphonate

G. O Adewuyi<sup>1\*</sup>, O. Olubomehin<sup>2</sup> and A. W Ayanniyi<sup>1</sup>

<sup>1</sup>Department of Chemistry, University of Ibadan, Nigeria.

<sup>2</sup>Department of Chemical Science, Olabisi Onabanjo University, Ogun State, Nigeria.

Accepted 14 December, 2009

A simple, fast and reproducible method for the determination of proguanil using high performance liquid chromatographic with UV/Fluorescence detection is described. Proguanil was derivatised to its corresponding derivative [(N<sup>1</sup>-(4-chlorophenyl)-N<sup>5</sup>-(1-methyl ethyl) imidocarbonimideamide-N-benzoxazole]. The derivatisation reaction was conducted in methanol at 60°C using sodium benzoxazole-2-sulphonate under alkaline conditions. The resulting derivative was extracted with chloroform after which the extract was observed under UV lamp at 254 nm before TLC and HPLC analysis. Similarly, the derivatisation process was adapted for derivatisation of proguanil in urine sample. The reaction proceeded smoothly and rapidly. The extraction process was not cumbersome and eliminated the need for costly extraction and evaporation equipments. The resulting derivative fluoresced intensely under UV lamp. Direct HPLC analysis of the reaction mixture was found possible without interferences from excess reagent and endogeneous compounds like ammonium salts. The derivative eluted in less than seven minutes thus making the method suitable for routine use. The calibration plot was linear over the concentration range. A correlation regression of the order of 0.94 was obtained from the calibration curve which indicated a strong relationship between the instrument response and the concentration of proguanil. The discussion also summarizes the derivatisation chemistry that have not being fully explored to date but may find utility in future development of highly sensitive analytical methods for biguanide drugs.

**Key words:** Proguanil, derivatisation, fluoresced, endogeneous, biguanide.

## INTRODUCTION

Proguanil, a biguanide derivative of pyrimidine is the most active of a series of synthetic aryl biguanides compounds tested for antimalarial activity in the mid 1940s (Curd et al., 1945). Proguanil was demonstrated to be both efficacious and non toxic hence its wide use as a prophylactic anti-malarial (Wattanagoon et al., 1987). The use of proguanil in the prophylaxis and treatment of malaria has increased recently due to the emergence of chloroquine resistant *Plasmodium falciparum*. The use of proguanil in combination with other antimalarial drugs has also been reported to possess synergic toxicity on the malaria parasite (Gozal et al., 1991; Bouchaud et al., 2000).

The pharmacokinetic characteristics of a drug have been implicated in the mechanism through which parasites develop resistance to it (Moody et al., 1982). The elucidation of the pharmacokinetic characteristic of proguanil is therefore necessary if its continued use and potency is not to be forfeited. The determination of proguanil, titrimetrically (Stagg, 1947; Gallo et al., 1955) gravimetrically (Green and Babru, 1972) and colorimetrically (Meagraith et al., 1946) and microbially (Smith et al., 1961) are lacking both in specificity and sensitivity and are therefore of no use for the pharmacokinetic study on proguanil. There are however a few chromatographic methods with the advantage of specificity and sensitivity. The pioneering chromatographic work by Moody et al. (1982) utilized reverse phase ion pairing high performance liquid chromatographic technique for the separation of proguanil and

\*Corresponding author. E-mail: [adewuyio@yahoo.co.uk](mailto:adewuyio@yahoo.co.uk).

its two major metabolites cycloguanil and 4-chloro phenyl biguanide. A detection limit of 60 ng/ml of sample volume was achieved with the method. Edstein (1986) in his work achieved a better sensitivity of 25 ng/ml sample volume.

The modifications made to the methods described by Moody et al. (1982) included the extraction procedure and a change in the ion pairing agent from sodium lauryl sulphate to pentane sulphonic acid. Kelly and Fletcher (1986) also made slight modifications to the liquid extraction procedures of Moody et al. (1982) and the ion pairing agent to achieve improved sensitivity. Taylor et al. (1987) however utilized solid phase extraction procedure in the pretreatment of samples but retained the sodium lauryl sulphate ion pairing agent. Other works by Taylor et al. (1990), Bergqvist and Hosptadius (2000), Kolawole et al. (1995), Bergqvist et al. (1998), either aimed at the determination of proguanil when used in combination with other antimalarial drugs or from very small sample volumes with little or no modifications above described methods.

It must however be stated that there are some inconsistencies in the analytical data on proguanil from the above methods hence the report that proguanil has limited pharmacokinetic data in literature by the WHO informal consultation on the use of antimalarial drugs (2001). It is in the light of this that this work sets to develop a method which is specific sensitive and reproducibly determines proguanil. This is hoped to be achieved by derivatizing proguanil with sodium benzoxazole-2-sulphonate and the resulting derivative determined by high performance liquid chromatography with UV/Fluorescence detection.

## MATERIALS AND METHODS

### Reagents and chemicals

Reagents and chemicals used in this study include proguanil hydrochloride powder (ICI), proguanil hydrochloride tablet (Astra Zeneca) high performance liquid chromatography (HPLC) grade acetonitrile (BDH), HPLC grade methanol (sigma) sodium benzoxazole-2-sulphonate (synthesized).

### Instrumentation

The chromatographic apparatus consisted of a Cecil 1200 series 1000 high performance liquid chromatograph. The analytical column was ODS hypersil C<sub>18</sub>, 5 µm particle size in 250 mm length x 4.6 mm I.D stainless steel column. The mobile phase consisted of acetonitrile/ water 65:35 v/v. The flow rate was 1 ml/min and column temperature was maintained at 25 °C with detection at 254 nm.

### Preparation of sodium benzoxazole- 2 -sulphonate

Sodium benzoxazole-2-sulphonate was synthesized following the procedure described by Idowu and Adewuyi (1993). Using the procedure, a mixture made of O-aminophenol (15.0 g), 75 ml methanol, a solution of 10 g potassium hydroxide in 20 ml distilled water and 50 ml carbon disulphide was refluxed for 2.5 h after

which another 30 - 50 ml portion of carbon disulphide was added. After refluxing for about 30 min, the mixture was allowed to cool to room temperature and decolourising charcoal was added. After refluxing for another 10 - 15 min, the mixture was filtered through fluted filter paper. The filtrate was distilled to dryness and the residue dissolved in 100 ml of water and the solution treated with a solution of 30 ml glacial acetic acid mixed with equal volume of water. 2-Mercaptobenzoxazole was immediately precipitated as a whitish solid. The solid was filtered and dried in air.

2-Mercaptobenzoxazole (5.0 g) was mixed with about 15 g phosphorus pentachloride and mixture heated on boiling water bath for 1 h. The mixture was then transferred to a hot plate and refluxed for another 1.5 h. After cooling to room temperature, the mixture was treated with 50 ml of a 25% (w/v) solution of sodium sulphate. After the vigorous effervescence had stopped, 50 ml sodium sulphate solution was further added and the mixture refluxed for 2 h by heating on a hot plate. The mixture was then filtered hot through fluted filter paper. As the solution cooled, needle shaped crystals of sodium-benzoxazole-2-sulphonate were formed.

### Preparation of benzoxazole derivative of proguanil

Proguanil (0.1 g) was dissolved in 2.0 ml of methanol and added to a solution of 0.1 g sodium benzoxazole- 2 -sulphonate in 5.0 ml of methanol and 1.0 ml 5 M sodium hydroxide solution added. After warming in a water bath (60 °C) for 5 min (Figure 1), the solution was observed under U.V. light. After cooling, the mixture was extracted with 5 ml chloroform. The aqueous layer was removed with a Pasteur pipette and the chloroform dried with anhydrous sodium sulphate. The chloroform extract was then transferred to another test tube and the chloroform removed under a stream of nitrogen. The residue was then reconstituted in 1 ml methanol and examined under the UV light before thin layer chromatography (TLC) and HPLC analysis.

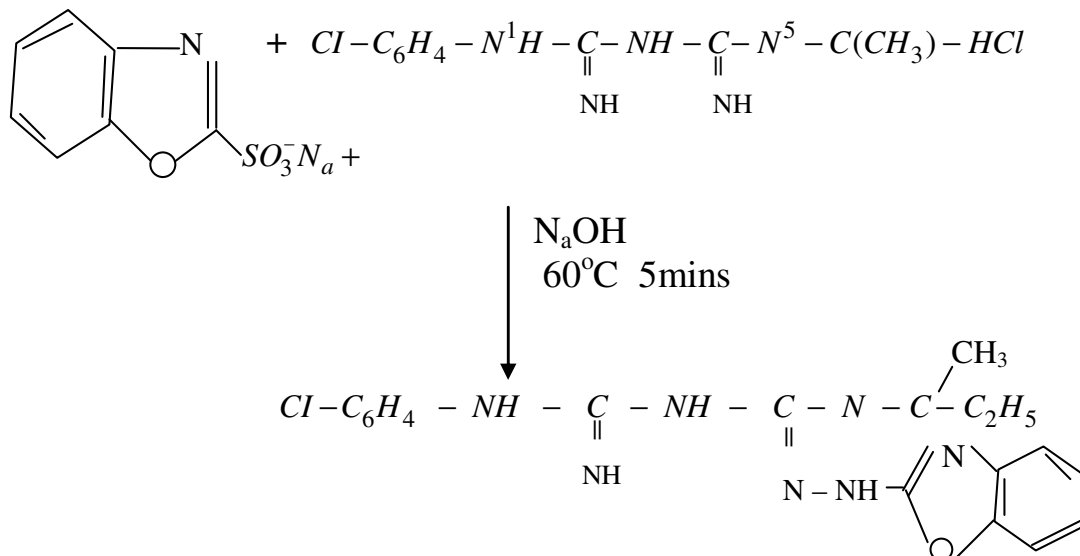
### Analytical procedure

**Preparation of stock solution of proguanil:** Stock solution containing 100 µg/ml proguanil was prepared in distilled water.

**Calibration curve in blank urine sample:** Using four extraction tubes, 1 ml blank urine samples were introduced, after which, varying amounts of the stock solution (100 µl/ml) of proguanil were added to give calibration ranges between 0 - 6 µl/ml for proguanil . 100 µl of sodium benzoxazole- 2 -sulphonate solution in methanol was added to each of the test tube, the mixture were rendered alkaline with 2 M NaOH (0.5 ml) and whirlmixed for 1 min then warmed at 60 °C for 5 min. The mixture was allowed to cool after which 3 ml chloroform was added to each of the samples and the tubes were centrifuge at 1500 rpm for 5 min. The chloroform layer was aspirated into another tube and dried over sodium sulphate crystals. The extraction with chloroform was repeated twice. The pooled extract was evaporated to dryness in a water bath at 40 °C .The residue was reconstituted in 100 µl of methanol and whirl-mixed before 20 µl was injected onto the HPLC .

## RESULTS AND DISCUSSION

An ideal derivatisation reagent for HPLC with UV or fluorescence detection should possess certain characteristics. Its UV or fluorescence characteristics should be completely different from that of the derivative so that excess reagent will not interfere with the detection of the



**Figure 1.** Reaction of sodium benzoxazole-2-sulphonate with proguanil.

derivative. Alternatively, if the reagent and the derivative have similar spectral characteristic, their chromatography behaviours should be widely different to allow easy separation of the excess reagent from the derivative. If this cannot be achieved by simple solvent extraction, the reagent should react readily with the analyte without any complicating side reactions such as undesirable hydrolytic decomposition which is observed with some reagents during derivatisation reactions with proguanil. Also, the derivative should be stable to light. Furthermore, it should be possible to carry out the derivatisation reaction in a variety of solvents and solvent combination that are to be encountered during the intended chromatographic applications. These are important requirements if the reagent is to be applicable to on-line pre or post column derivatisation in a possible automation of the analytical method. Proguanil consist of a biguanide with two substituents p-chloro benzene and isopropyl groups at N<sup>1</sup> and N<sup>5</sup> positions. The basic structure of proguanil is responsible for its polar nature which is similar for all biguanide drugs. Biguanides are therefore strong bases. The polar nature creates problem of isolation and determination as is experienced for proguanil. However, the reaction of proguanil with a water soluble fluorescent reagent like sodium benzoxazole-2-sulphonate is expected to produce a derivative that fluoresced intensely and can be determined with ease. The observation of the derivatised solution under a UV light gave an intensified blue colouration which was not observed with proguanil. This was an indication that fluorescence derivative resulted from the reaction.

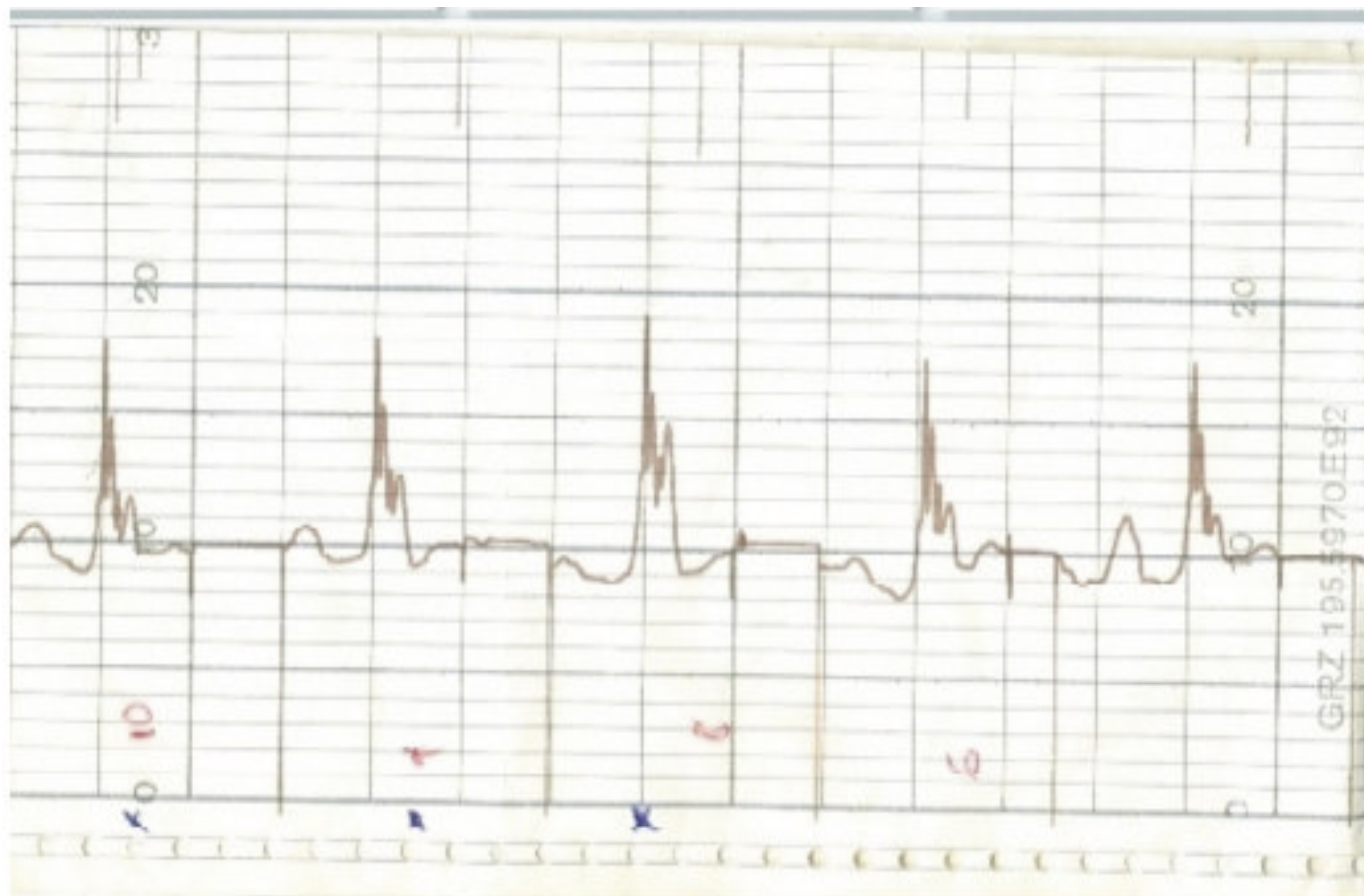
Thin layer chromatography was done to establish the chromatographic behaviours. The derivative was detectable at low microgram level by TLC followed by examination of the plate under UV light. The RF of derivative

was 0.48; while sodium benzoxazole-2-sulphonate gave RF of 0.98. The retention value for benzoxazole was consistent with the observation of Idowu and Adewuyi (1993). The derivative being a large molecule had a retention value of 0.48 which was expected and in agreement with previous observation. This chromatographic behaviour is note worthy because it revealed that excess derivatising reagent would not interfere with detection.

### Determination of proguanil in urine

Chromatographic separation was carried out under gradient condition at ambient temperature on a reversed phase column (ODS Hypersil) with UV/FL wavelength at 254 nm. A mobile phase consisting of acetonitrile - water (65:35 v/v) at a flow rate of 1.0 ml/min was found to be suitable. A representative liquid chromatographic separation of the derivatized proguanil in urine is reported in Figure 2. The figure revealed that the reagent and solvent did not interfere with the analysis. Also, there were no interferences from endogenous compounds in the urine sample used in the study, thereby facilitating accurate determination of the drug. The method gave good recovery for derivatized proguanil (> 90%) in urine (Table 1). This is an improvement over previous method reported (Eldstein, 1986; Taylor et al., 1987) and this report corresponds with the observation of Ebeshi et al. (2005). The HPLC method was reproducible with coefficient of variation (which is a measure of precision) being less than 10% in urine (Table 2).

Therefore, the method exhibits good precision and sensitivity. The calibration curve using solution with known concentration of derivatized proguanil was linear with correlation coefficient of not less than 0.94 (Figure 3) in



**Figure 2.** A representative liquid chromatographic separation of derivatised proguanil in urine.

**Table 1.** Precision of analytical method for derivatized proguanil in urine.

Conc. ( $\mu\text{ml}$ )	Number of samples (n)	Coefficient of variation %			
		PGB1	PGA2	PGA3	PGA4
0	4	4.26	5.35	4.20	3 - 28
2	4	3.20	4.82	3.82	4.14
4	4	5.28	3.45	2.86	2.46
6	4	6.12	4.23	5.14	4.34

PGB1 = Blank (no proguanil); PGA2, PGA3, PGA4 = concentration ranges of proguanil.

**Table 2.** Peak area ratio and correlation coefficient for derivatized proguanil in urine.

Sample	Peak area ratio	$y = 11.45x - 0.9774$ $R^2 = 0.9418$
PGB1	2.15394	
PGA2	22.38840	
PGA4	74.44965	

the urine sample used in the study. A similar trend was observed by Ebeshi et al. (2005) in their study in which the calibration curve for proguanil was linear with correlation of not less than 0.9.

The method may be further developed to address the challenge of simultaneous determination of proguanil and its major metabolites due to the presence of a primary amino functionality on both cycloguanil and 4-chlorophenylbiguanide that could be derivatised with sodium-

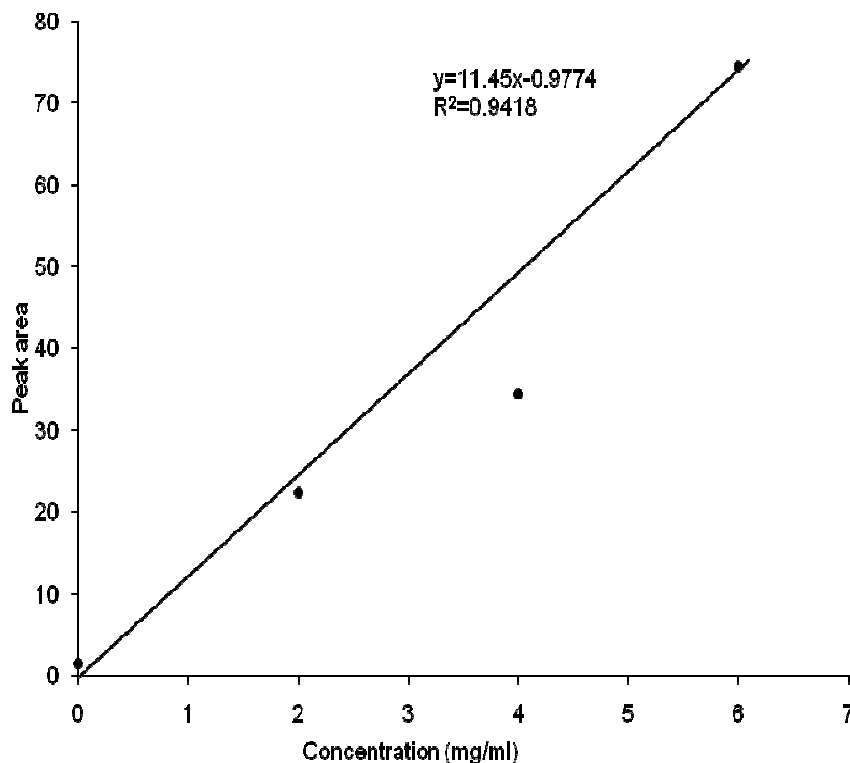


Figure 3. Calibration curve for derivatised proguanil in urine sample.

benzoxazole-2-sulphonate and hence reduce the paucity of methods with such capacity (Ebeshi et al., 2005).

## REFERENCES

- Bergqvist Y, Hopstadius C (2000). Simultaneous Separation of Atovaquone, Proguanil and Its Metabolites on A Mixed Mode High Performance Liquid Chromatography Column. *J. Chromatogr. B.* 741: 189-193.
- Bergqvist Y, Funding L, Kaneko A, Krysen B, Leek T (1998). Improved Method for the Simultaneous Determination of Proguanil and Its Metabolites by High Performance Liquid Chromatography and Solid Phase Extraction of 100 $\mu$ L Capillary Blood Samples Dried on Sampling Paper. *J. Chromatogr. B.* 719: 141-149.
- Bouchaud O, Monlum E, Munanza K, Fontamet A, Scott T, Goetschel A, Chulay JD, Le Bras J, Davis M, Le Bras M, Coulad JP, Gentilini M (2000). Atovaquone Plus Proguanil versus Halofantrine for the Treatment of Imported Acute Uncomplicated Plasmodium Falciparum Malaria in Non-Immune Adults: Randomised Comparative Trial. *Am. J. Trop. Med. Hyg.* 63. 5, 6: 274-270.
- Curd FHS, Davey DG, Rose FL (1945). Studies on Synthetic Anti-Malaria Drugs X - Some Biguanide Derivatives as New Types of Anti-Malarial Substances with both Therapeutic and Causal Prophylactic Activity. *Ann. Trop. Med. Parasitol.* 39: 208-216.
- Ebeshi UB, Obodozie OO, Bolaji OO, Ogunbona AF (2005). Sensitive High Performance Liquid Chromatographic Method for the Determination of Proguanil and Its Metabolites, Cycloguanil and 4-Chlorophenyl Biguanide in Biological Fluids. *Afr. J. Biotechnol.* 4(8): 856-861.
- Edstein MD (1986). Simultaneous Measurement of Proguanil and Cycloguanil in Human Plasma by High-Performance Liquid Chromatography. *J. Chromatogr.* 380: 184-189.
- Gallo U, Nastasi A, Novocic L (1955). Determination of Proguanil in Non-aqueous Solvents. *Bull-Chim. Farm.* 94: 85-89
- Gozal D, Hengy C, Fadat G (1991). Prolonged Malaria Prophylaxis with Chloroquine and Proguanil (Chrologuanide) in a non-immune resident population of an endemic area with high prevalence of chloroquine resistance. *Antimicrob. Agents Chemother.* 35(2): 373-376.
- Green I, Barbu S (1972). The Dosage of chloroguanidine HCl. *Farmacia (Bucharest).* 15(1): 1-6.
- Idowu OR, Adewuyi GO (1993). Sodium benzoxazole-2-sulphonate: A Derivatisation reagent for the analysis of amines and amino acids by HPLC with fluorescence and UV detection. *J. Liquid Chromatogr.* 17: 3773-3791.
- Kelly JA, Fletcher KA (1986). High Performance Liquid Chromatographic Method for the Determination of Proguanil and Cycloguanil in Biological Fluids. *J. Chromatogr.* 381: 464-471.
- Kolawole JA, Taylor RB, Moody RR (1995). Determination of Proguanil and its Metabolites in Small Sample Volumes of Whole Blood Stored on Filter Paper by High Performance Liquid Chromatography. *J. Chromatogr. B.* 674: 149-154.
- Meagraith BG, Tottey MM, Adams ARD, Horner Andrews WH, King JD (1946). The Absorption and Excretion of Paludrine In The Human Subject. *Ann. Trop. Med. Parasitol.* 40: 495-506
- Moody RR, Selkirk AB, Taylor RB (1982). High performance liquid chromatograph of proguanil, cycloguanil and 4-chlorophenylbiguanide using hydrophobic pairing-ion and its application to serum assay. *J. Chromatogr.* 182: 359-367.
- Smith CC, Ihrig J, Menne T (1961). Anti-Malarial activity and metabolism of biguanides I.; metabolism of chlorguanide triazine in rhesus monkeys and man. *Am. J. Trop. Med.* 10: 694-703.
- Stagg H (1947). Determination of N-(p-Chloro)-N-Isopropyl Biguanide. *J. Pharm. Pharmacol.* 1: 391-394.
- Taylor RB, Behrens R, Moody RR, Wangbooskul J (1990). Assay method for simultaneous determination of proguanil, chloroquine and other major metabolites in biological fluids *J. Chromatogr.* 527: 490-497.
- Taylor RB, Moody RR, Ocheke NA (1987). Determination of proguanil and its metabolites cycloguanil and 4-chlorophenyl biguanide in

plasma, whole blood and urine by high performance liquid chromatography. *J. Chromatogr.* 416: 384-399.

Wattanagoon Y, Taylor RB, Moody RR, Ochckpe NA, Looareesuwan S, White NJ (1987). Single dose pharmacokinetics of proguanil and its metabolites in healthy subjects. *Br. J. Clin. Pharmacol.* 24: 175-780.

World Health Organization (2001). Report of WHO informal consultation on the use of antimalarial drugs.