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Full Length Research Paper

Sensitive high performance liquid chromatographic method for the determination of proguanil and its metabolites, cycloguanil and 4-chlorophenylbiguanide in biological fluids

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A new simple, sensitive, cost-effective and reproducible high performance liquid chromatographic (HPLC) method for the determination of proguanil (PG) and its metabolites, cycloguanil (CG) and 4-chlorophenylbiguanide (4-CPB) in urine and plasma is described. The extraction procedure is a simple three-step process that has eliminated the need for costly extraction and evaporation equipment. The mobile phase consisted largely of buffer, making the method cheap to run. The calibration plots were linear over the concentration range up to 3.0 µg/ml PG, CG and 4-CPB in urine and concentration range up to 1000 ng/ml in plasma. The correlation coefficients (r) were of the order of 0.99 and above for PG and 4-CPB and 0.98 for CG. The ion pair method was carried out on a 5 µ reversed-phase C-18 column, using perchlorate ion as the counter ion and ultra violet detection at 254 nm. The method was reproducible with coefficient of variation for PG, CG and 4-CPB, being less than 10% in urine and plasma. PG was well resolved from its metabolites, CG and 4-CPB, and the internal standard, pyrimethamine. The limit of detection of PG was 10 ng/ml and the recovery was greater than 90% in urine and plasma. The analytical method therefore, exhibits good precision and sensitivity and is one of the few methods that can detect PG and the two metabolites CG and 4-CPB. The analytical method developed in this study was used to determine PG bioavailability after rectal administration in humans.

Key words: Proguanil, HPLC, metabolites, biological fluids.

INTRODUCTION

Proguanil, a synthetic biguanide derivative of pyrimidine, is widely used in chemoprophylaxis of malaria. It is chronically administered for malaria prophylaxis in sickle cell patients in Nigeria. The emergence of chloroquine resistant Plasmodium falciparum in our environment has led to a resurgence of interest in the use of proguanil when daily prophylaxis of malaria is indicated. In addition proguanil has found use in combination with other drugs such as atovaquone and dapsone in the treatment of resistant cases of falciparum malaria (Looareesuwan et al., 1996). This renewed interest in the use of proguanil makes it necessary to elucidate fully the pharmacokinetics of the drug in biological fluids, which requires highly sensitive, accurate and specific method of analysis.

The various methods described for the determination of proguanil and its metabolites in the body includes colorimetric, microbiological and chromatographic methods. The colorimetric and bioassay method of analysis as described by (Maegraith et al., 1946; Watkins et al., 1987) lacked specificity and sensitivity. They are, therefore, of little value in pharmacokinetic studies.

A chromatographic method reported for the analysis of proguanil in the biological fluid is the reversed phase, ion-pair high performance liquid chromatographic (HPLC) technique developed by Moody et al. (1980) for the assay of proguanil in serum. Specific quantitation was obtained but the assay sensitivity was inadequate for the

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determination of the metabolites.

Edstein (1986) reported ion-pair reversed phase technique using a 30 x 3.9 mm id column packed with 10 µ/m -Bondapak C18. A detection limit of 25 ng/ml was reported for cycloguanil and proguanil, but the method was unable to detect the second metabolite, 4-CPB, in the sample plasma obtained from a human volunteer dosed with 200 mg proguanil. Kelley and Fletcher (1986) also reported the use of cyanopropyl packed columns with an aqueous mobile phase containing ammonium formate as the ion-pairing agent. These HPLC methods described above were unable to detect 4-CPB in the presence of proguanil in plasma samples.

With more emphasis on off-column sample pretreatment of biological samples prior to HPLC analysis as a necessary panacea to low level detection of drugs in biological fluid, Taylor et al. (1987) employed liquid-liquid extraction procedure. It was observed that the use of hexane as the sole extracting solvent resulted in poor recoveries for cycloguanil and 4-CPB. They made a modification of the solvent composition, the solvent mixture composed of hexane and isoamyl alcohol (4:1) was used. There was a resultant low recovery of both cycloguanil and proguanil as compared to earlier report by Edstein (1986) but CPB was detected.

Other workers, who used HPLC method in analyzing proguanil and major metabolites in biological fluids, utilized the modification of the method of Taylor et al. (1987). They could still not resolve the problem of low recovery of the metabolites (Chiluba et al., 1987; Wattanagoon et al., 1987; Watkins et al., 1987).

Due to some of these inconsistencies on the analytical data of proguanil and inter laboratory differences, the report of WHO informal consultation on the use of antimalarial drugs (2001) adjudged proguanil to have limited pharmacokinetic data in literature. This study therefore, set out to develop a simple, cost-effective, sensitive and accurate method that can measure accurately the concentration of proguanil and its metabolites in different biological fluids.

MATERIALS AND METHODS

Chemicals, reagents and apparatus

Proguanil hydrochloride powder (ICI), proguanil hydrochloride tablets (Zeneca Pharmaceutical, Ltd), cycloguanil powder (ICI), 4-chlorophenylbiguanide powder (ICI), pyrimethamine powder (BDH), ammonium acetate (BDH), perchloric acid 60% w/w (BDH), diethyl ether (BDH), HPLC grade acetonitrile (Sigma Aldrich), HPLC grade methanol (Fisher scientific) and acetone (BDH). 220V Ultra sonicator (Branso), whirlmixer (Scientific Industries Inc.), table centrifuge {Gallenkamp}, precision pipettes (Eppendorf), Pasteur pipettes and extraction tube

Preparation of stock solutions

Stock solutions containing 100 µg/ml proguanil, cycloguanil and 4-chlorophenylbiguanide were prepared in distilled water, while pyrimethamine, which was used as the internal standard, was prepared in methanol.

Chromatographic conditions

The Liquid chromatographic system used consisted of a Cecil 1100 series instrument (Cecil Instrument, Cambridge, England) made up of binary pumps fitted with a gradient mixer (Cecil instrument) with a system purge and a variable wavelength (200-800 nm) ultraviolet-visible detector model CE1200 (Cecil instrument) with a 18 µL flow cell. Injection was by a Rheodyne model 7725 valve (Cotati, California, U.S.A.) fitted with a 20 µL loop. The detector output is linked to a CTX Computer (made in Thailand) via a brain-box inter-phase (Cecil Instrument), which transforms signals from the detector to the computer that eventually records the chromatograms. The computer system is connected to an LX 300 printer (Epson). The column used was a Hypersil ODS (C-18) 5µm particle size and 250 x 4.6 mm I.D, reversed phase stainless steel (Alltech). A mobile phase consisting of methanol : acetonitrile : 0.5% ammonium acetate (40:5:55) containing 75 mM/L perchloric acid was pumped through the column at a flow rate of 1.2 ml/min. The pH of the mobile phase was 2.2, and the chromatogram was run at ambient temperature.

Analytical procedure

Calibration curve in urine: 1 ml blank urine samples were placed in extraction tubes; varying amounts of the stock solutions (100 µg/ml) of proguanil, cycloguanil and 4-chlorophenylbiguanide were added to give calibration curves between 0.1 – 3 µg/ml for proguanil, 4-chlorophenylbiguanide and cycloguanil. 20 µL of the stock solution of the internal standard, pyrimethamine (100 µg/ml), was added to each tube. The urine samples were rendered alkaline with 2 M NaOH (0.5 ml) and whirlmixed for 1 min. Then 3 ml ether was added to each of the samples and whirlmixed for 1 min after which the tubes were centrifuged at 1500 rpm for 10 min. The upper organic layer was aspirated into another tube. The extraction with ether was repeated twice. The pooled extract was evaporated to dryness in a water bath at 40^{o}C. The residue was reconstituted in 100 µL of methanol and whirlmixed before injecting 20 µL onto the HPLC. The peak area ratio was plotted against the concentration of each of the compounds injected. The regression analysis was carried out with the aid of a computer.

Calibration curve in plasma: To 1 ml blank plasma samples placed in centrifuge tubes, varying amounts of the stock solution of proguanil, cycloguanil and 4-chlorophenylbiguanide were added to give a concentration between 0.05 - 1.0 µg/ml for the three compounds. 10 µL of the internal standard solution was added to each tube. The samples were extracted with ether under alkaline conditions as described for urine. The residue was reconstituted in 100 µL of methanol, whirlmixed before 20 µL was injected onto the HPLC system.

Determination of drug and metabolites in biological samples

Analysis of test urine samples: To 1 ml of urine sample in a centrifuge tube, 20 µL of the internal standard was added. PG and its metabolites, CG and 4-CPB were extracted under alkaline conditions as previously described. The residue was reconstituted in 100 µL methanol, whirlmixed before 20 µL was injected onto the HPLC.

Analysis of test plasma samples: To 1 ml of plasma sample in extraction tube, 10 µL of the internal standard was added. The

Table 1. Precision of analytical method in urine.

Precision period	Conc. µg/ml	No. of samples (n)	Coefficient of Variation (%)		
			PG	CG	4-CPB
Within-day	0.5	4	5.78	4.65	3.26
	2.0	4	2.47	4.65	3.4
Day-to-day	0.5	4	4.05	4.36	2.16
	2.0	4	1.90	5.97	4.49

Table 2. Precision of analytical method in plasma.

Precision period	Conc. µg/ml	No. of samples (n)	Coefficient of Variation (%)		
			PG	CG	4-CPB
Within-day	0.5	4	6.14	8.33	6.15
	2.0	4	4.29	9.74	2.78
Day-to-day	0.5	4	3.19	2.95	2.38
	2.0	4	3.03	8.04	4.30

extraction and reconstitution followed as described above before injecting 20 µL onto the HPLC.

Precision studies in plasma and urine

Within-run precision studies: Two sets, each set consisting of four centrifuge tubes, were used. Each tube in the first set contained 1 ml of blank sample (urine or plasma) spiked with the stock solution of the three compounds to give a concentration of 0.5 µg/ml. The second set also contained 1 ml of blank sample (urine or plasma) spiked with stock solution of the three compounds to give a concentration of 2 µg/ml of each. All the samples were then spiked with 20 µL of the internal standard solution in case of urine and 10 µL in the case of plasma. Extraction followed under alkaline conditions as earlier described. The residue was reconstituted in 100 µL methanol, whirlmixed before 20 µL was injected onto the HPLC. The coefficient of variation of each set was computed.

Day-to-day Precision: The procedure above was followed but a sample for each set was analyzed daily for 4 days.

Recovery studies in plasma and urine

Two sets, each set consisting of four centrifuge, tubes were used. Each tube in the first set contained 1 ml of blank sample (urine or plasma) spiked with the stock solution of the three compounds to give a concentration of 0.5 µg/ml. The second set contained the same amount of blank sample spiked to give a concentration of 2 µg/ml for the three compounds. All the samples were then spiked with 20 µL of the internal standard solution in the case of urine and 10 µL in the case of plasma. Extraction followed under alkaline conditions as described earlier. The residues were reconstituted in 100 µL methanol and whirlmixed before 20 µL was injected onto the HPLC. In two other tubes, the stock solutions were diluted in such a way as to give 0.5 µg /ml and 2 µg/ml for the three compounds. The two tubes were spiked with 20 µL of the internal standard solution in case of urine and 10 µL in the case of plasma. To determine the recovery, the peak area ratio of the extraction method and direct injection method were compared.

RESULTS AND DISCUSSION

The HPLC method used for the analysis gave a good resolution of proguanil from its two metabolites, cycloguanil and 4-chlorophenylbiguanide and the internal standard (pyrimethamine). There was no interference from endogenous compounds in all the biological samples used in the study, thus facilitating accurate determination of the drug and its metabolites. The method also gave good recoveries for proguanil (>90%) and one of the metabolites, CPB (>88%), in urine and plasma (Tables 1 and 2). The recoveries for PG and CPB were lower but still acceptable in plasma being 87 and 79%, respectively. But the analytical method, on the other hand, showed moderate sensitivity to the major metabolite, cycloguanil, leading to its moderate recovery (about 70% or less) in urine and plasma. Previous workers, who have attempted to quantify proguanil and its metabolites using HPLC method, reported poor recoveries of cycloguanil and low detection of CPB (Eidstein, 1986; Taylor et al., 1987). The poor recovery of cycloguanil may not be unconnected with the solvent systems employed in the extraction procedure by those workers. In this study, the choice of diethyl ether as the extracting solvent resulted in some improved recovery of cycloguanil compared to previous reports in literature.

The HPLC method was reproducible with coefficient of variation (which is a measure of precision) for proguanil and its metabolites, CG and CPB, being less than 10% in urine and plasma (Tables 3 and 4). The method, therefore, exhibits good precision and sensitivity. The analytical method employed in this study is one of the few methods that can detect proguanil and the two metabolites despite the moderate recovery of CG.

Table 3. Results of recovery studies in urine.

Conc. (µg/ml)	No. of Samples (n)	Percentage recovery		
		PG	CG	4-CPB
0.5	4	94.4 ± 3.4	61.9 ± 4.4	109.5 ± 3.6
2.0	4	90.3 ± 5.2	66.5 ± 6.3	88.5 ± 4.1

Table 4. Results of recovery studies in plasma.

Conc. (µg/ml)	No. of Samples (n)	Percentage recovery		
		PG	CG	4-CPB
0.5	4	90.3 ± 6.6	66.2 ± 7.9	86.8 ± 4.7
2.0	4	70.9 ± 6.3	70.9 ± 6.3	79.2 ± 7.5

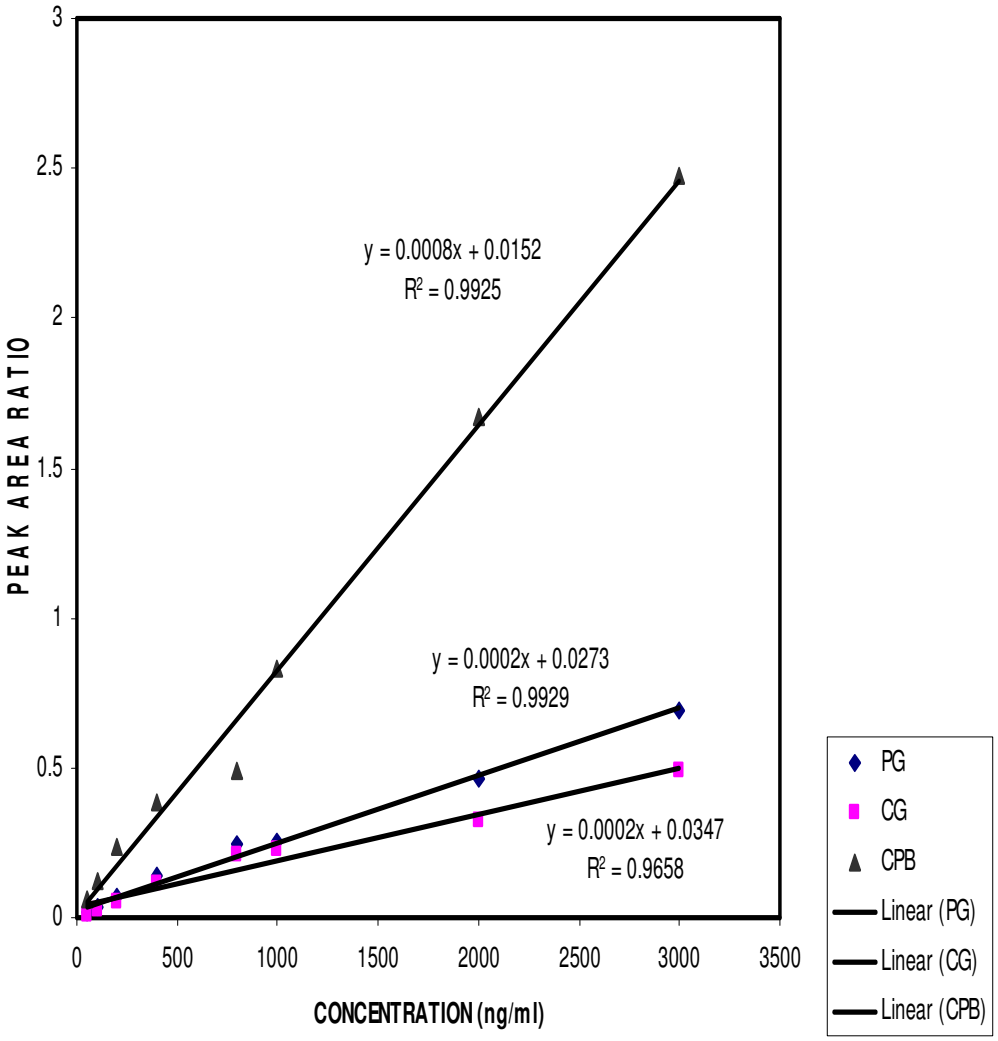

Figure 1. Calibration curves for proguanil (PG), cycloguanil (CG) and 4-chlorophenylbiguanide (4-CPB) in urine.

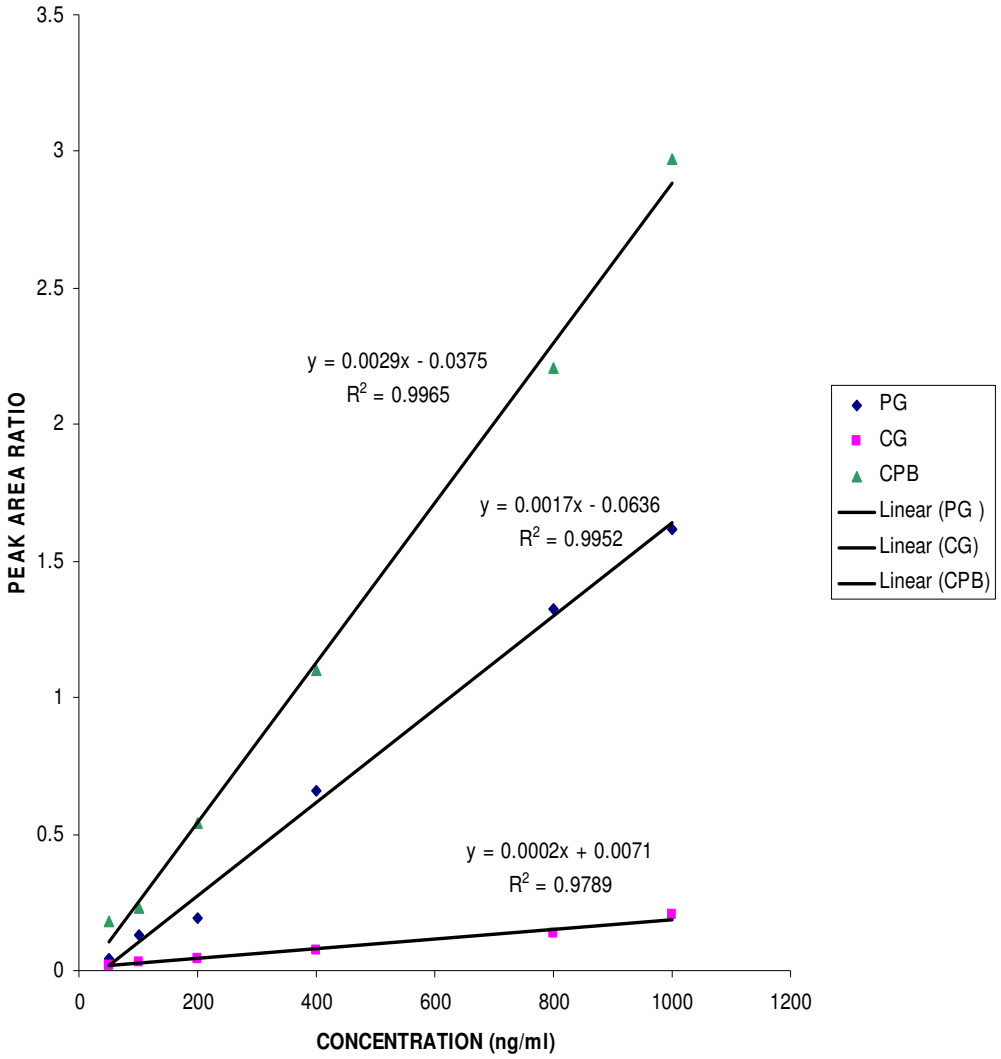

Figure 2. Calibration curves for proguanil (PG), cycloguanil (CG) and 4-chlorophenylbiguanide (4-CPB) in plasma.

The calibration curve for proguanil was linear with correlation coefficient of not less than 0.99 (Figures 1 and 2) in all the biological samples used in the study. The calibration curves were also linear for the two metabolites, cycloguanil (R^2>0.96) and 4-chlorophenylbiguanide (R^2>0.99), in all the biological fluids used in the study.

This method was used to study proguanil bioavailability from suppository in comparison with tablet formulation. The detection of measurable level of proguanil and metabolites in urine and plasma following the administration of the proguanil suppository and tablet to all the ten volunteers is an indication that the method is selective and sensitive.

The lower limit of quantitation of PG in urine and plasma, which was 10 ng/ml made it possible to monitor the unchanged drug for the whole duration of sample collection when this method was used to study PG bioavailability from suppository formulation in comparison to the tablet formulation. The detection of the measurable levels of PG, CG and 4-CPB in urine and plasma following the administration of the PG suppositories and tablets to all volunteers who participated in the study was a clear indication that this method is selective and sensitive.

REFERENCES

Chiluba EM, Fletcher KA, Price AH (1987). The pharmacokinetics of proguanil in human subjects following a single oral dose. Afr. J. Med. Sci. Br. 16: 43-46.

Eidstein MD (1986). Simultaneous measurement of Proguanil and Cycloguanil in human plasma by High Pressure Liquid Chromatography. J. Chromatogr. Amsterdam, 380: 184-189.

Kelly JA, Fletcher KA (1986). High Pressure Liquid Chromatography method for the determination of proguanil and cycloguanil in biological fluids. J. Chromatogr. Amsterdam. 381: 464-471.

Looareesuwans S, Viravan C, Webster HK, Kule DE, Hutchinson DB, Canfield CJ (1996). Clinical studies of atovaquone alone or in combination with other antimalaria drugs in the treatment of acute uncomplicated malaria in Thailand. Am. J. Trop. Med. Hyg. 54: 62 – 68.

Maegreath BG, Tottey MM, Adam ARD, Andrews WH, King JD (1946). the absorption and extraction of proguanil in human subject. Ann. Trop. Med. Parasitol. 40: 493-506.

Moody RR, Selkirk AB, Taylor RB (1980). High Performance Liquid Chromatography of proguanil, cycloguanil and 4-chlorophenyl-biguanide using a hydrophobic pairing ion and its application to serum assay. J. Chromatogr. 182: 359-367.

Taylor RB, Moody RR, Ochekpe NA (1987). Determination of proguanil and its metabolites, cycloguanil and 4-chlorophenylbiguanide in plasma, whole blood and urine by High Pressure Liquid Chromatography. J. Chromatogr. Amsterdam. 416: 394-399.

Watkins WM, Chulay JD, Sixsmith DG, Spencer HC, Howells RE (1987). A preliminary pharmacokinetic study of the antimalarialdrugs, proguanil and chlorproguanil. J. Pharm. Pharmacol. 39: 261-265.

Wattanagoon Y, Taylor RB, Moody RR, Ochekpe NA, Looareesuwan S, White NJ (1987). Single dose Pharmacokinetics f proguanil and its metabolite in healthy subjects. Br. J. Clin. Pharmacokinet. 24: 775-780.

World Health Organisation (2001). Report of a WHO Informal Consultation on the use of antimalarial drugs.