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Note

High-performance liquid chromatographic method for the determination of proguanil and cycloguanil in biological fluids

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Proguanil hydrochloride (Paludrine, ICI) is widely used as a prophylactic agent against malaria. Two 100-mg tablets, administered once daily, is now the recommended regimen.

Proguanil undergoes cyclic oxidation by hepatic microsomal oxidases of the cytochrome P-450 system to form its active metabolite, cycloguanil [1]. It is the metabolite, rather than the parent drug that is thought to possess the prophylactic activity [2]. Since metabolism plays an important part in its activity, more information is required on the kinetics of proguanil and its main metabolite. The only pharmacokinetic data available [3] were obtained using colorimetric assays [4, 5] which were non-specific and lacked sensitivity. A simple, reproducible, specific and sensitive method for the estimation of proguanil and its metabolite, cycloguanil, is therefore required for measuring the nanogram levels found in plasma after prophylactic doses. Moody et al. [6] developed a high-performance liquid chromatographic (HPLC) method capable of detecting the biguanides in serum but with a detection limit in the region of 60 ng ml^{-1}. This method was insufficiently sensitive since the low in vivo concentrations of metabolite could not be determined. The purpose of the present study was to develop a rapid, selective and sensitive HPLC method for the simultaneous estimation of proguanil and cycloguanil in plasma and urine.

The following system employing reversed-phase, ion-pair chromatography was found to be suitable. Ammonium formate was used as the ion-pairing reagent. Metoprine, used by Jones and Ovenell [7] for the measurement of pyrimethamine was chosen as the internal standard as its structure resembled that of proguanil and cycloguanil. The structures are given in Fig. 1.

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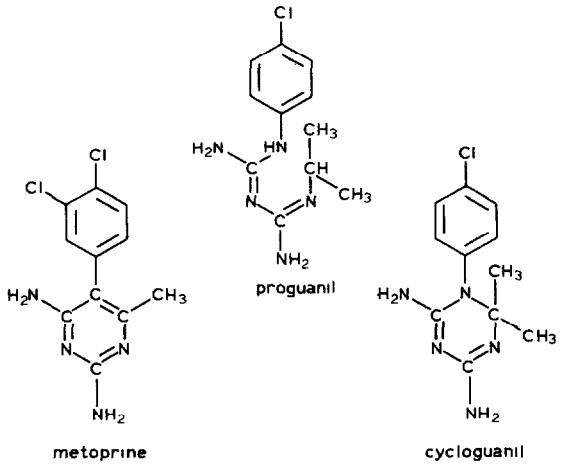

Fig. 1. Structures of proguanil, cycloguanil and metoprine, the internal standard.

EXPERIMENTAL

Chemicals and standards

Acetonitrile was HPLC grade (Rathburn Chemicals, Walkerburn, U.K.). Ammonium formate and sodium hydroxide were of analytical quality (BDH

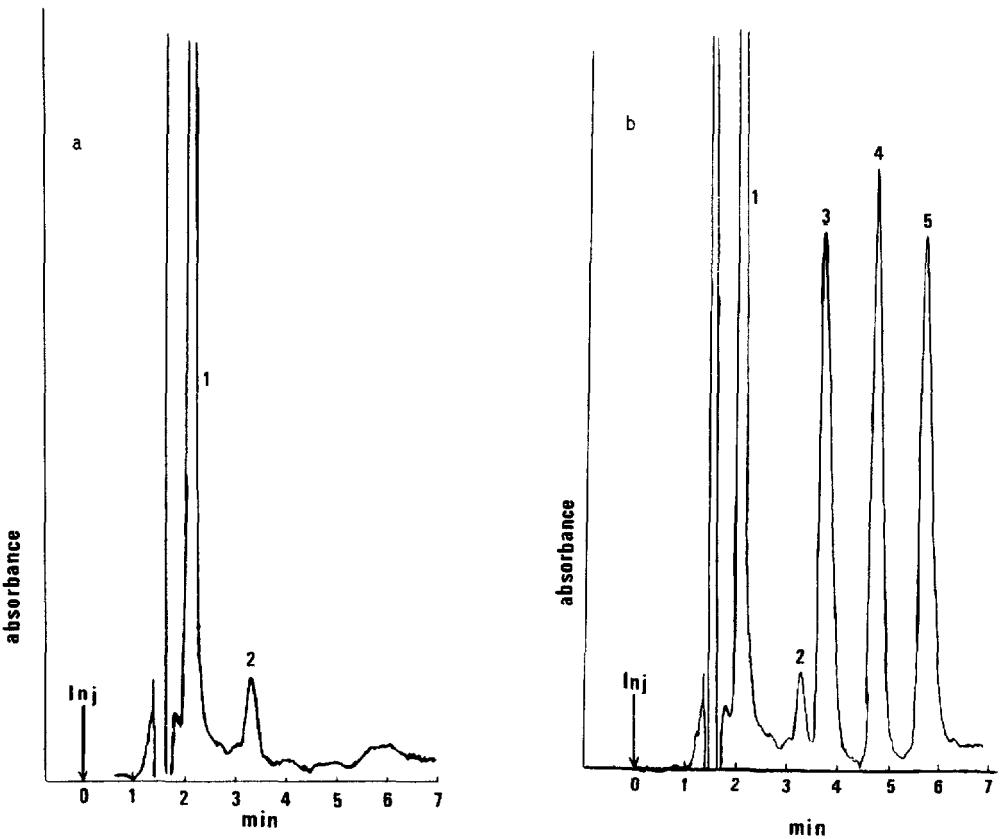

Fig. 2.

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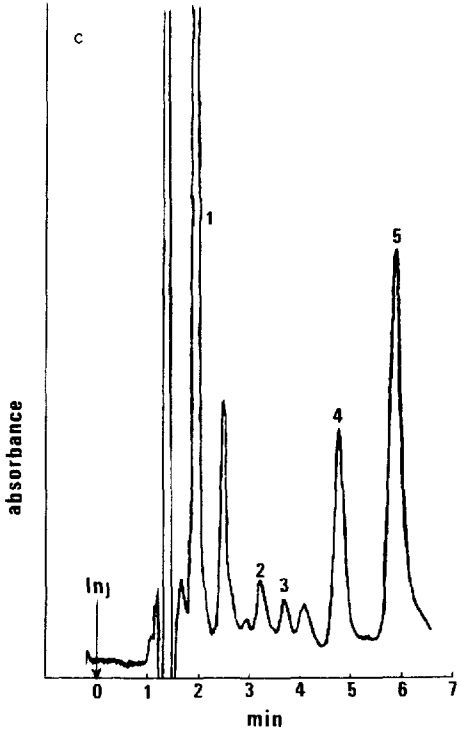


Fig. 2. Chromatograms of plasma extractions. (a) Human plasma; (b) human plasma spiked with cycloguanil, proguanil and metoprine; (c) plasma from subject administered 200 mg proguanil daily (analysis showed levels of 118 ng ml^{-1} proguanil and 34 ng ml^{-1} cycloguanil). Conditions as in text. Peaks: 1 = solvent front; 2 = unknown plasma constituent; 3 = cycloguanil; 4 = proguanil; 5 = metoprine, internal standard.

Chemicals, Liverpool, U.K.). Chloroform was also of analytical grade (May and Baker, Dagenham, U.K.). Paludrine tablets, containing 100 mg proguanil hydrochloride, and cycloguanil were obtained from Imperial Chemical Industries (Macclesfield, U.K.). Metoprine [2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine] was donated by Wellcome Research Labs. (Beckenham, U.K.). A stock standard solution of proguanil was prepared by grinding a Paludrine tablet in 2 ml of 1 *M* hydrochloric acid and diluting with water. The solution was centrifuged at 1500 *g* for 5 min to separate the insoluble tablet excipients before dilution to a concentration of 1 mg ml^{-1} (as base). Cycloguanil and metoprine were initially dissolved in methanol then diluted with water to prepare stock standard solutions. All standard solutions were stored at 4°C.

Instrumentation and chromatographic conditions

The liquid chromatographic system comprised of a Laboratory Data Control Constametric III pump and a Rheodyne Model 7120 high-pressure injection valve with a 50-μl loop, linked to a Laboratory Data Control Spectromonitor III UV absorbance detector operated at 252 nm at a sensitivity of 0.01 a.u.f.s. The column was a 25 cm × 4.6 mm I.D., 5 μm particle size, Spherisorb nitrile

column, S5CN (Phase Separations, Queensferry, U.K.). The detector output was connected to a Kipp and Zonen BD8 flat-bed recorder.

The mobile phase consisted of acetonitrile—methanol—water (9:2:89, v/v) containing 0.05 *M* ammonium formate (pH 4.0) and was degassed using a Decon, FS 100 ultrasonic bath (Decon Ultrasonics, Hove, U.K.). The flow-rate was 2.0 ml min^{-1} and the system was operated at ambient temperature. Representative chromatograms are shown in Fig. 2. The retention times for cycloguanil, proguanil and metoprine were 3.9, 5.0 and 6.1 min, respectively.

Extraction procedure

An aliquot (250 μl) of the internal standard, metoprine (1 μg ml^{-1}), was added to 1 ml of each plasma sample in a 15-ml screw-capped tube followed by 1 ml of 1 *M* sodium hydroxide and 5 ml of chloroform. The contents of the tubes were gently agitated on a rotary mixer for 30 min. After centrifugation at 1500 *g* for 5 min, the aqueous phase was removed and 3 ml of the organic phase were transferred to a conical glass tube. The organic phase was evaporated to dryness at room temperature using a gentle stream of nitrogen. The residue was dissolved in 60 μl of mobile phase by vortex-mixing and an aliquot (20 μl) injected onto the chromatographic column. Duplicate extractions and injections were performed.

Calibration

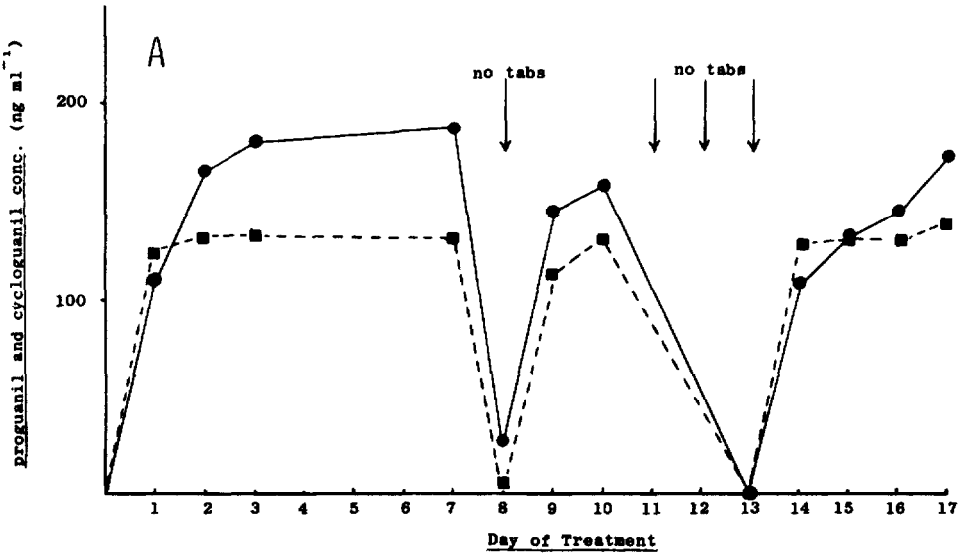
Calibration curves were prepared by analysing a 1.0-ml plasma sample spiked with known amounts of proguanil and cycloguanil. The concentration range studied was 25—500 ng ml^{-1} for each compound. Peak-height ratios of proguanil and cycloguanil to the internal standard were plotted against concentrations. A calibration curve was constructed before samples were analysed. Subsequently, two standard extractions from spiked plasma were performed along with each group of samples analysed daily. Standard results were collected and correlated to form a combined standard line for the analysis of a batch of samples. Such calibration curves of concentration against peak-height ratios for each compound were linear with correlation coefficients of not less than 0.996. The regression equations for typical calibration curves were $y = 0.419x + 1.920$, $r = 0.998$ ($n = 8$) for proguanil and $y = 0.227x + 1.354$, $r = 0.996$ ($n = 8$) for cycloguanil.

Urine analysis

Urine was diluted in the ratio 1:10 to 1:50 with water depending on the collection time and volume of the sample. Aliquots (1 ml) were analysed as described for plasma with modified standard solutions. Standard solutions consisted of control urine spiked with proguanil and cycloguanil to give concentrations of 0.5—5.0 μg ml^{-1} and the concentration of the internal standard was 10 μg ml^{-1}.

Recovery

The recovery of proguanil and cycloguanil was determined by comparing peak-height ratios of each compound to the internal standard extracted from spiked plasma (range 25—500 ng ml^{-1}) with ratios obtained by direct injection



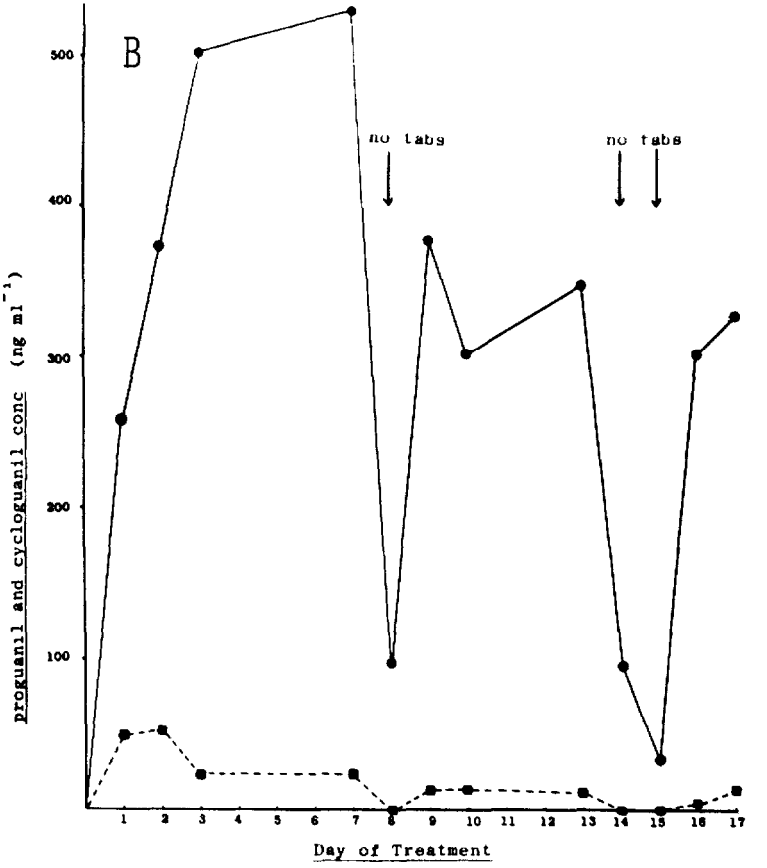


Fig. 3. Proguanil (●——●) and cycloguanil (■ - - - ■) plasma levels 4 h after 200 mg of Paludrine daily in (a) a male and (b) a female volunteer.

of standard aqueous solutions containing equivalent concentrations of the compounds. The overall recovery over this concentration range was 97 ± 5% for proguanil and 65 ± 4% for cycloguanil (n = 3).

The method was validated in plasma over a range from 25 to 500 ng ml^{-1} and 0.5 to 5.0 μg ml^{-1} in urine, although plasma levels of 10 ng ml^{-1} could be determined from the plasma standard curve. The coefficient of variation between duplicates was < 4% at levels of 25 ng ml^{-1} and < 2% for higher concentrations.

Proguanil and cycloguanil were found to be stable for at least three months when plasma or urine samples were stored at −20° C prior to analysis.

Application to preliminary studies in humans

Two volunteers (a male and a female) were administered two Paludrine tablets (200 mg proguanil hydrochloride) daily as a single dose. Venous blood samples were collected in heparin, 4 h after daily dosage for analysis of plasma levels. Doses were missed for periods of one, two and three days so that the rates of decrease in proguanil and cycloguanil concentrations could be measured. Urine samples were collected over 24 h, after a plasma steady state had been reached, usually after three days of administration.

RESULTS

Plasma concentrations for the male and female volunteers are shown in Fig. 3 and are similar to the results of Maegraith et al. [3]. The results indicate that a steady state is reached after the third day of treatment. When daily doses are not taken, levels fall sharply but do not disappear completely until at least two or three doses are missed. The results of the male and female volunteer were surprisingly different. Plasma and urine levels of proguanil were higher in the female but levels of cycloguanil were lower. Fig. 4 shows the urine levels of both volunteers. Over 24 h the female volunteer excreted 90 mg of proguanil and 8 mg of cycloguanil, while the male excreted 30 mg of proguanil and 42 mg of cycloguanil. The results of the male volunteer agreed generally with those of the large group of Army personnel discussed below.

Random blood samples (n = 150) were taken from each of two groups of Army personnel taking 200 mg Paludrine daily for malaria prophylaxis. Samples were collected between 2 and 6 h (group 1) and between 2 and 12 h (group 2) after dosing to monitor compliance. After eliminating those plasma results which showed obvious non-compliance, the mean values for the personnel in group 1 were 169.3 ± 67.1 and 83.1 ± 41.6 ng ml^{-1} for proguanil and cycloguanil, respectively, and in group 2 204.94 ± 66.06 and 63.04 ± 35.88 ng ml^{-1}.

DISCUSSION

The HPLC method described allows the selective analysis of proguanil and its metabolite cycloguanil in plasma and urine at prophylactic concentrations. Metoprine proved to be an excellent internal standard; it had a reproducible extraction efficiency of > 95% (very similar to proguanil) and a suitable

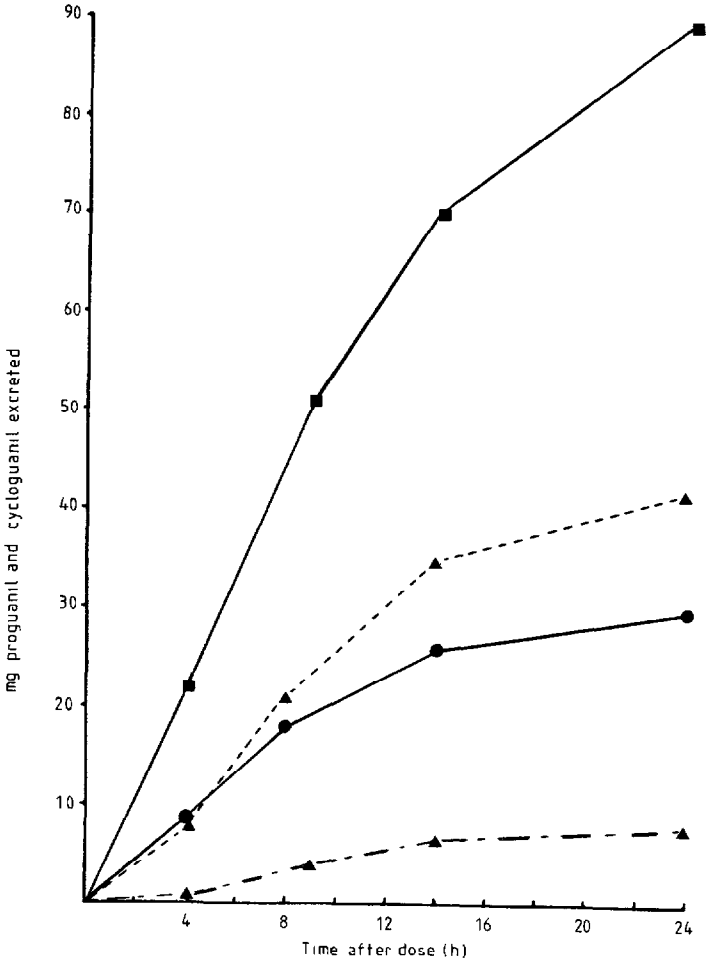


Fig. 4. Urine excretion of proguanil (solid lines) and cycloguanil (broken lines) in a male (●—●, ▲----▲) and a female (■—■, ▲- —-▲) volunteer

retention time. Methanol was an essential component of the mobile phase for the resolution of drug and metabolite from other endogenous substances which did not interfere with the assay. The advantages of this HPLC method are ease of sample preparation, speed of analysis and a detection limit that allows simultaneous analysis of the metabolite, unlike a previously published HPLC method [6].

The method was successfully applied to the analysis of levels in volunteers taking Paludrine for malaria prophylaxis. Results from these studies show that levels fall relatively quickly when doses are missed and indicate that drug compliance can be easily monitored. Results from the female volunteer were different from those of the male volunteer, even when weight differences were accounted for. This observation could be clinically important because cycloguanil, the metabolite, is the active form of the drug [2]. Factors that influence metabolism must therefore be considered e.g. diet, absorption, sex differences, weight and interactions with other drugs. The female volunteer

was also taking an oral contraceptive steroid (OCS). Further investigations have already been carried out in female volunteers to study the effects of OCS on the metabolism of proguanil. The results will be described elsewhere.

The plasma levels obtained in the two groups of Army personnel studies were similar to those in the initial male volunteer. Although most subjects appeared to comply with the recommended regimen, 15.07% in group 1 and 12.66% in group 2 showed unusually high or low levels of either proguanil and/or cycloguanil, suggesting poor compliance.

CONCLUSION

A selective and sensitive HPLC procedure for the simultaneous estimation of proguanil and cycloguanil in plasma and urine has been described. The method was used to measure prophylactic levels in volunteers given the drug, and is now being used in our laboratory for routine monitoring of plasma levels and for pharmacokinetic studies of Paludrine.

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