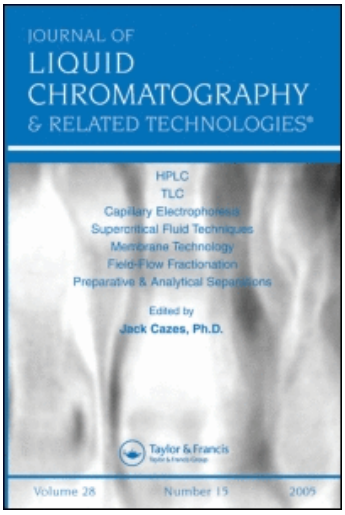

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A SIMPLE HPLC ASSAY FOR PROGUANIL AND ITS ACTIVE METABOLITE CYCLOGUANIL: APPLICATION TO OXIDATION PHENOTYPING

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ABSTRACT

A simple high-performance liquid chromatographic (HPLC) method for simultaneous determination of proguanil and its active metabolite cycloguanil in human urine has been developed. Quinine sulphate was used as the internal standard. The assay uses a reversed phase C18 microbore column (2 mm I.D. x 10 cm) packed with 3 μm ODS Hypersil. The chromatographic separation was achieved by using an isocratic mobile phase comprising acetonitrile-aqueous phosphate buffer (10:90, v/v) containing 200 mM sodium dodecyl sulphate adjusted to pH 2. The mobile phase was pumped at 0.4 ml/min. The eluant was monitored by a UV detector operating at 254 nm. The assay was based on an organic extraction with 1-hexanol/ether (40: 60, % v/v) and then back-extracted into a small volume of acidic aqueous solution before injection onto the HPLC column. With this procedure coefficients of variation were less than 8%. The detection limit was 0.5 μg/ml of urine. The method is simple, sensitive, selective and allows for routine analysis of urine samples in the genetic drug oxidation phenotyping study in ethnic population.

INTRODUCTION

Proguanil (PG) is an antimalarial drug commonly used for malaria (i.e., *Plasmodium falciparum*) prophylaxis. Although it is not widely used in Southeast Asia regions, the metabolism of proguanil is important as the drug itself is not

pharmacologically active and must be metabolised by P450 enzyme to form the active metabolite, cycloguanil (1). The metabolism of proguanil, which is catalysed by a particular cytochrome P450 isoenzyme *(CYP2C19)*, has also been found to exhibit an evidence of polymorphism (1-4). The genetic drug oxidation polymorphism of proguanil has been studied in a few ethnic populations. Two oxidation phenotypes have been identified. In Caucasian population, most subjects are extensive metabolisers (EMs) of proguanil, whereas a minority of subjects are poor metabolisers (PMs). For instance approximately 3% of a Caucasian population were identified as PMs of proguanil, whose the ability to convert proguanil (PG) to its active metabolite, cycloguanil (CG) was markedly reduced (1). An increasing number of studies have demonstrated that genetic oxidation polymorphism of proguanil shows pronounced inter-ethnic variability. In contrast to the Caucasian population, the incidence of PMs of proguanil appears to be considerably higher in non-Caucasian populations. A study carried out in 100 healthy Kenyan adults showed that 35% were identified as PMs (5). A recent study in a Thai population reported the incidence of proguanil PMs to be 18% (6). The difference in ability of individuals to convert this prodrug to the active metabolite, cycloguanil, could have important clinical consequences with respect to the therapeutic success of this antimalarial prodrug.

A number of high-performance liquid chromatographic (HPLC) methods have been developed (7-11). These methods allow for the simultaneous determination of proguanil and its metabolite cycloguanil in biological fluids. However, most of these assays involved many extraction steps and some used solid phase extraction as sample preparations before HPLC analysis which is less economic and time consuming. The present report describes a simple and sensitive HPLC method for simultaneous quantitation of proguanil and its metabolite cycloguanil in human urine. The applicability of this procedure is demonstrated by the analysis of urine samples collected from volunteers who participated in a study of drug oxidation phenotyping which was aimed to determine the PM incidence of proguanil, in an ethnic population.

MATERIALS AND METHODS

Reagents and Chemicals

Proguanil hydrochloride and cycloguanil were gift from Major M D Edstein, Australian Army Malaria Research Unit, New South Wales, Australia. Quinine

sulphate (as an internal standard) was purchased from Sigma Chemical Co (St Louis, MO, USA). Sodium dodecyl sulphate (SDS), orthophosphoric acid, HPLC-grade acetonitrile, 1-hexanol and diethyl ether were purchased from BDH Chemicals Ltd (Poole, England). All chemicals used were analytical grade. Glassware was cleaned and silanized with 0.05% Aquasil® (Pierce Chemical Co., Rockford, IL, USA) before use. Water was double glass distilled and MilliQ® filtered.

Standard Solutions and Internal Standard

Stock solutions (100 μg/ml) of proguanil and cycloguanil were prepared in HPLC water and stored at -20°C until required. These solutions was found to be stable for at least 2 weeks at -20°C. Urine standard solutions of proguanil and cycloguanil for the calibration curves were prepared by proper dilution of the stock solutions with drug-free urine so that concentrations of 0.5, 1, 2.5, 5, 10, 20 and 50 μg/ml were obtained. The internal standard solution of quinine sulphate (40 μg/ml) was prepared in HPLC-grade water. This solution was protected from light and stored at -20°C until required; it was found to be stable for at least 2 month.

Sample Preparation

To 2 ml of urine sample in a silanized centrifuge tube, 150 μl of 4 M NaOH and 200 μl of internal standard solution (40 μg/ml quinine sulphate) were added. The contents were then extracted with 6 ml of 1-hexanol/ether (40: 60, % v/v) by mechanically shaking for 25 minutes. The samples were centrifuged for 10 minutes at 1500 g (at 4°C) to separate the phases. The organic layer was transferred to a clean tapered glass centrifuge tube containing 250 μl of 0.1% orthophosphoric acid. The mixture was shaken for 25 minutes and centrifuged (1500 g, 4°C) for 10 minutes. The organic layer was aspirated and discarded. The aqueous extract was transferred to the autosampler plastic vials and 50 μl was injected into the HPLC column.

Chromatographic Conditions

The HPLC system consisted of a Model 250 Perkin Elmer LC pump (Perkin Elmer Corporation, Norwalk, CT, USA) equipped with a Jasco Model AS-950 autosampler (Jasco Corporation, Tokyo, Japan). A variable wavelength ultraviolet detector (Spectroflow 757, Kratos Analytical Instruments, Ramsey, NJ, USA) operating at 254 nm was used with a setting of 0.01 a.u.f.s. The chromatographic response was recorded by a Shimadzu R3A integrator (Shimadzu, Kyoto, Japan).

A microbore HPLC column (2 mm I.D. x 10 cm) packed with a reversed-phase C18 material, 3 μm ODS Hypersil (Phenomenex, CA, USA) was used. The column efficiency was over 3000 plates per 10 cm. Analysis of the samples of proguanil and cycloguanil was performed using a mobile phase consisting of an acetonitrile-aqueous phosphate buffer (10 mM Na_2HPO_4) mixture (10:90, v/v) containing 200 mM sodium dodecyl sulphate (SDS) and adjusted to pH 2 with orthophosphoric acid. The flow rate of the mobile phase was 0.4 ml/min (back pressure approximately 1800 psi). Chromatographic separations were performed at room temperature.

Recovery

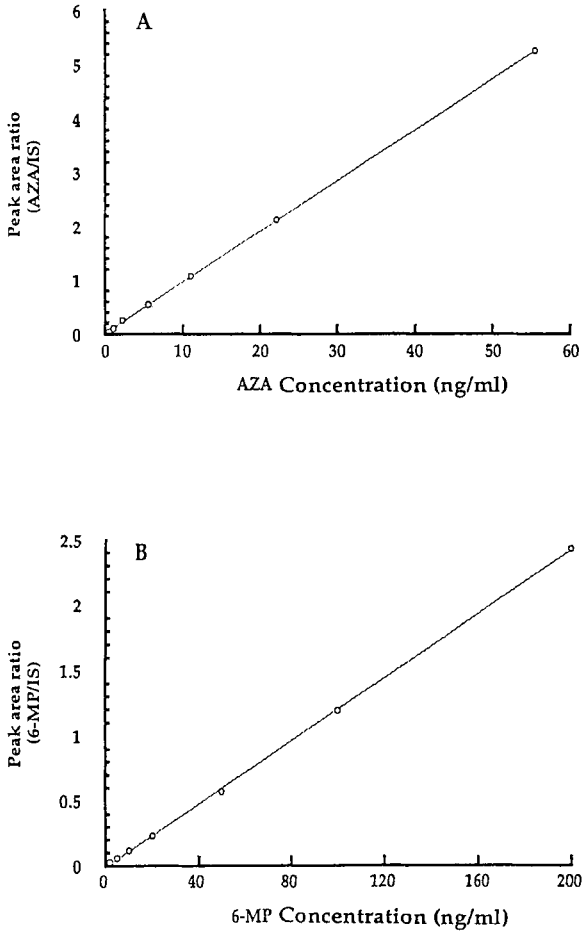
The assay recovery of proguanil and cycloguanil from urine samples was determined at 0.5 and 20 μg/ml. Absolute recovery was calculated by comparing the peak heights from 5 extracted urine samples with those obtained by direct injection of the pure drug standards. The absolute recovery of the internal standard (quinine) was assessed using the same procedure.

Calibration Curve

Standards corresponding to 0, 0.5, 1, 2.5, 5, 10, 20 and 50 μg/ml of proguanil and cycloguanil were prepared in drug-free urine. The sample analysis was performed as described above and standard curves were run daily. Quantitation is based on peak height ratios (proguanil or cycloguanil/internal standard). An unweighted least squares regression line was fitted to each individual calibration curve.

Proguanil Oxidation Phenotyping Study

The study protocol was approved by the Southern Regional Health Authority Ethics Committee (Otago), Dunedin, New Zealand. The subjects were 43 unrelated, healthy Maoris who were all born in New Zealand, aged from 18 - 35 years. On the study day, urine was voided immediately before they receiving an oral dose of 200 mg proguanil hydrochloride (Paludrine® tablets, ICI New Zealand). Urine was collected for the following 8 hours. The total urine volume was recorded and an aliquot was kept frozen at -20°C until analysis. Proguanil (PG) and its active metabolite cycloguanil (CG), were measured by the HPLC procedure described above. Individuals with urinary concentration (PG/CG) ratios greater than 10 were classified as PMs of PG (1).



4. Calibration curve for the assays of AZA (A) and 6-MP (B) in human plasma.

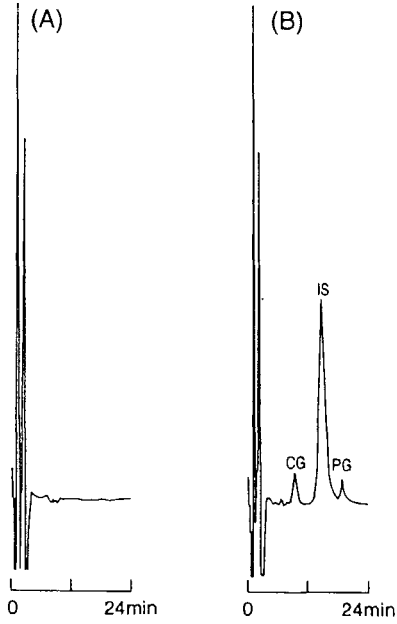


FIGURE 1

Typical chromatograms of extracts of human urine samples: (A) blank urine; and (B) urine spiked with 0.5 μg/ml cycloguanil and 0.5 μg/ml proguanil.
Peaks: CG = cycloguanil; PG = proguanil; IS = internal standard (quinine).

The within-day (within-run) reproducibility and accuracy of the proguanil and cycloguanil assay are presented in Table 1. At all concentrations studied the C.V. was less than 8%. These results indicate good precision of the assay. The C.V. of the assay at a concentration of 0.5 μg/ml, was 7.7% for proguanil and 6.4% for cycloguanil with good accuracy (Table 1). These C.V. values are lower than the generally accepted C.V. of 20% for minimum quantifiable concentration (MQC). Thus, the MQC or the detection limit of sensitivity for this assay was assigned at 0.5 μg/ml.

Urine samples stored at -20°C for up to 2 months showed no signs of decomposition and there was no difference in the urinary concentrations of proguanil and cycloguanil between the fresh samples and the stored urine samples ($n = 6$, $p > 0.2$). This indicates that proguanil and cycloguanil are stable under these storage conditions for at least 2 months.

TABLE 1

Within-day Reproducibility and Accuracy of the Assay for Proguanil and Cycloguanil in Human Urine Samples

Spike Concentration (μg/ml)	Observed Concentration[1] (μg/ml)	C.V. (%)	Accuracy[2] (%)
Proguanil			
0.5	0.52 ± 0.04	7.7	104
20	21.0 ± 1.1	5.2	105
Cycloguanil			
0.5	0.47 ± 0.03	6.4	94
20	19.8 ± 0.9	4.5	99

[1] Results given are mean ± S.D. (n = 5)

[2] Accuracy (%) = $\frac{\text{observed concentration}}{\text{spiked concentration}} \times 100$

The present method was used to determine the proguanil and cycloguanil concentrations in urine samples collected from healthy volunteers who participated in a study of the proguanil oxidation phenotyping. Examples of chromatograms obtained from a poor metaboliser (PM) and an extensive metaboliser (EM) of proguanil are shown in Figure 2. Of 43 Maori volunteers phenotyped, 3 subjects were identified as PMs of proguanil. This represents 7% prevalence of the proguanil poor metaboliser phenotype in the Maori population. Full details of this study on the evidence of poor metabolisers of proguanil in the New Zealand Maori population were published elsewhere (12). Although the detection limit of sensitivity (0.5 μg/ml) for the present assay was not as low as the other previous reports, we found that it provided sufficient sensitivity for the purpose of phenotyping. None of the urine samples analysed had a concentration of proguanil and cycloguanil below the detection limit. Some urine samples in fact needed to be diluted before analysis as the concentrations were too high.

In summary, a sensitive HPLC method has been described for the quantitative analysis of proguanil and its active metabolite cycloguanil in human urine. The procedures are simple and less time-consuming as the assay does not use solid

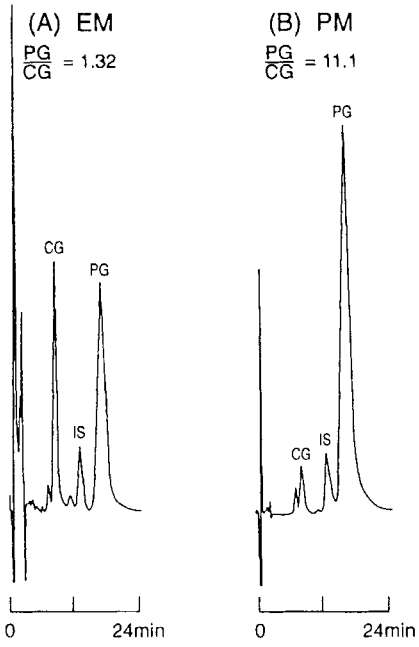


FIGURE 2

Examples of chromatograms of urine samples (0-8 hours) collected from an extensive metaboliser (A); and a poor metaboliser (B), after an oral dose of 200 mg proguanil hydrochloride (Paludrine® tablets, ICI New Zealand). Peak identification is as in Figure 1.

phase extraction. The method provides a sufficient sensitivity for the simultaneous determination of proguanil and cycloguanil. The assay has been shown to be suitable for use in studies of proguanil oxidation phenotyping in ethnic populations.

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