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Modified high-performance liquid chromatographic method to measure both dextromethorphan and proguanil for oxidative phenotyping

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Abstract

The activities of the polymorphic enzymes cytochromes P450 2D6 and 2C19 can be assessed by administering the probe drugs, dextromethorphan and proguanil, respectively. An existing high-performance liquid chromatographic technique, which measures dextromethorphan and its metabolites, has been modified to also measure proguanil and its polymorphic metabolite, cycloguanil in urine. Proguanil and cycloguanil are assayed in separate aliquots of urine to that used for dextromethorphan/dextrorphan as pretreatment with β-glucuronidase is required for the analysis of dextrorphan. To assay all four compounds a common extraction procedure is used and a single reversed-phase column and isocratic mobile phase with UV and fluorescence detectors connected in series are required. This technique is specific and sensitive for each analyte (limits of detection, dextrorphan/dextromethorphan/proguanil: 0.1 μg/ml, cycloguanil: 0.2 μg/ml). All assays are linear over the concentration ranges investigated (dextromethorphan/dextrorphan: 0.5–10 μg/ml, proguanil/cycloguanil: 1–20 μg/ml). The method described therefore uses laboratory resources very efficiently for all the assays required for hydroxylation phenotyping using proguanil and dextromethorphan. © 1997 Elsevier Science B.V.

Keywords: Dextromethorphan; Proguanil

1. Introduction

Due to their relative safety and availability, dextromethorphan (DM) (an antitussive) and proguanil (PG) (an antimalarial) have been used as probe drugs to assess the activity of the polymorphic enzymes cytochrome P450 2D6 (CYP2D6) [1] and cytochrome P450 2C19 (CYP2C19) [2]. The *O*-demethylation of DM to dextrorphan (DR) cosegregates with the CYP2D6 or debrisoquine/sparteine genetic polymorphism in drug metabolism [3], while the oxidation of PG to its active metabolite, cycloguanil (CG), co-segregates with the CYP2C19 or (*S*)-mephenytoin genetic polymorphism in drug metabolism [4]. The ratios of the urinary recoveries of the parent drugs (DM or PG) to the corresponding polymorphic metabolites over a defined time interval are used to classify an individual as either a poor (PM) or extensive metaboliser (EM) of CYP2D6 or CYP2C19. The polymorphisms are inherited independently and it is often of interest to know both the activity of cytochrome P450 2D6 and cytochrome P450 2C19 in the same individual. Recent studies have shown that DM and PG can be coadministered

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so that both hydroxylation phenotypes can be determined from the one urine collection [5,6]. The genetic basis of each polymorphism is understood [7–9], and although genotyping tests for CYP2D6 and CYP2C19 are now available, dextromethorphan and proguanil are still valuable investigative tools, as phenotyping provides an index of an individual's cytochrome P450 enzyme activity which is not revealed by genotype alone.

To measure the concentrations of DM, PG and their polymorphic metabolites in urine, chromatographic assays that are specific for each analyte are required. The techniques should have short sample run-times, be linear over the relevant concentration ranges and cost-efficient. Previously two separate assays have been required to determine both DM and PG phenotypes [5,6]. Dextromethorphan and DR concentrations in urine have been measured using a number of high-performance liquid chromatographic (HPLC) techniques with fluorescence detection [10–13]. Proguanil and CG concentrations have been measured by reversed-phase HPLC techniques with UV detection which employ expensive ion-pairing reagents and/or solid-phase extraction columns [14–16]. This paper describes a novel technique for the measurement of PG and CG in urine which is based on an existing method to measure urinary DM and DR [10] and which uses a common extraction technique for all four analytes. By using both ultraviolet absorption and fluorescence detectors in series and modifying the mobile phase described in the literature, both DM and DR as well as PG and CG can be measured using the single chromatographic system.

2. Experimental

2.1. Chemicals

Dextrorphan tartrate, 3-methoxymorphinan hydrobromide and 3-hydroxymorphinan hydrobromide were kindly supplied by Roche (Sydney, Australia). Proguanil hydrochloride, cycloguanil and chlorcycloguanil were gifts from ICI Pharmaceuticals (Melbourne, Australia). Pholcodine was a gift from Dr. John Lewis (Toxicology Unit, Royal North Shore Hospital, St. Leonards, Australia) and dextromethorphan hydrobromide and β-glucuronidase (*Helix pomatia*, type H-1) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile, methanol, triethylamine (all HPLC grade) and sodium carbonate (analytical grade) were purchased from BDH (Kilsyth, Australia). Hydrochloric acid, orthophosphoric acid (85%), chloroform, diethyl ether and propan-2-ol (all analytical grade) were purchased from Ajax (Auburn, Australia).

2.2. Chromatographic technique

The mobile phase, consisting of acetonitrile–triethylamine–double distilled water (6:0.12:93.88, v/v/v) adjusted to pH 3 using orthophosphoric acid, was pumped at a flow-rate of 1.3 ml/min using a 510 pump (Waters Associates, Millford, MA, USA) through a Spherisorb 5 μm CN 150×4.6 mm I.D. column (Alltech Associates, Deerfield, IL, USA) resulting in a back pressure of 9000 kPa. A 712 WISP (Waters Associates) was used to inject samples onto the column. A Lambda-Max Model 481 UV detector (Waters Associates) set at 238 nm detected PG, CG and chlorcycloguanil (internal standard). In series after the UV detector a Waters 474 scanning fluorescence detector (Waters Associates) was set at emission and excitation wavelengths of 275 and 302 nm, respectively to detect DR, DM and pholcodine (internal standard). An SP4600 Data-jet Integrator (Spectra-Physics, San Jose, CA, USA; attenuation 32) recorded the peak height output of the UV detector and a 3390A Hewlett Packard integrator (Hewlett Packard, Avondale, PA, USA; attenuation 4) recorded the peak height output of the fluorescence detector. The system was operated at room temperature.

2.3. Solutions

Aqueous stock solutions (1 mg/ml) of DM hydrobromide, DR tartrate and pholcodine were prepared in double distilled water (stored at 4°C) and methanolic solutions (1 mg/ml) of PG, CG and chlorcycloguanil solutions were prepared and stored at −20°C in polypropylene tubes (10 ml Sarstedt, Numbrecht, Germany) because these compounds adhere to glass [14]. Working solutions of the internal standards, pholcodine (250 μg/ml) and

chlorcycloguanil (20 μg/ml) were prepared in double distilled water and stored at 4°C.

2.4. Sample preparation

The pretreatment of urine for the measurement of DM, DR, PG and CG concentrations is outlined in Fig. 1.

2.4.1. CYP2D6 phenotyping

To measure the concentration of DM and DR in urine, a 0.5 ml sample was incubated overnight (approximately 16 h) in a tapered polypropylene tube with 0.5 ml β-glucuronidase (8000 U/ml in 0.2 *M* acetate buffer, pH 5) at 37°C. Pholcodine (50 μl of 250 μg/ml) was added prior to organic solvent extraction.

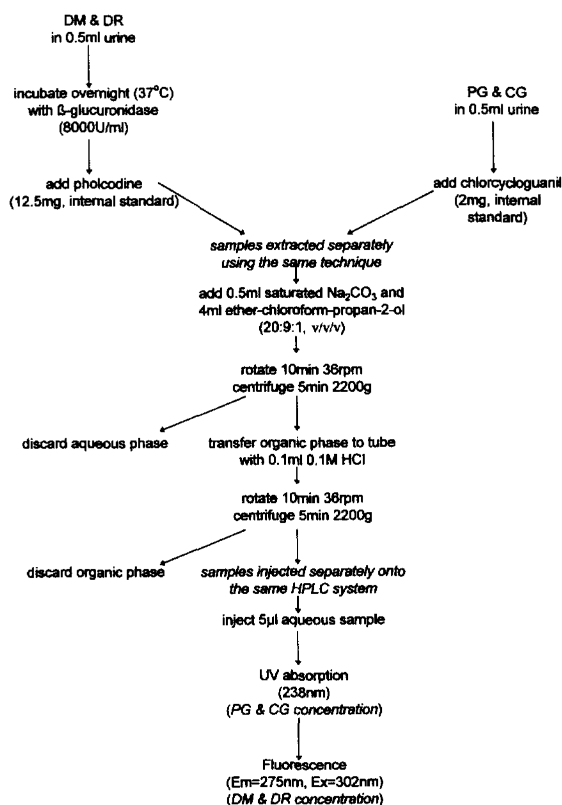

Fig. 1. Flow chart illustrating the preparation of urine samples and their analysis.

2.4.2. CYP2C19 phenotyping

To measure the concentration of PG and CG in urine, 50 μl of chlorcycloguanil (40 μg/ml) was added to 0.5 ml of urine in a tapered polypropylene tube prior to organic solvent extraction.

2.4.3. Organic solvent extraction

Incubated and non-incubated urine were extracted separately using the method of Chen et al. [10]. In brief, 0.5 ml of saturated sodium carbonate was added to the urine sample and the tube was vortexed. Four millilitres of diethyl ether–chloroform–propan-2-ol (20:9:1, v/v/v) was added and the tubes were rotated (36 rpm) for 10 min followed by centrifugation for 5 min at 2200 *g*. The organic layer was transferred to a tapered plastic tube containing 0.1 ml 0.1 *M* hydrochloric acid. Samples were rotated (36 rpm) for 10 min followed by centrifugation for 5 min at 2200 *g*. The organic phase was discarded and an aliquot (5 μl) of the aqueous phase was injected onto the HPLC column.

2.5. Calibration curves

Six standards prepared in drug-free urine over the concentration range 0.5–10 μg/ml for DM and DR and 1–20 μg/ml for PG and CG were extracted in duplicate as outlined above. Standard curves were estimated by linear regression of concentration added versus peak height ratios of the analyte to the internal standard.

2.6. Extraction efficiency

Urine samples to which 3 concentrations of DR (0.54, 5.4, 10.8 μg/ml), DM (0.49, 4.9, 9.7 μg/ml), PG (1.13, 11.3, 22.6 μg/ml) and CG (1.05, 10.5, 21.0 μg/ml) were added, were extracted ($n=6$) as described above. The peak heights of DR, DM, PG and CG were compared with the peak heights for the same amount of each analyte injected onto the HPLC column. The difference observed was the extraction recovery.

2.7. Precision, accuracy and limit of detection

To determine the within-day variabilities of the assays for all analytes, drug-free urine was spiked

with three concentrations of DR (0.54, 5.4, 10.8 μg/ml), DM (0.49, 4.9, 9.7 μg/ml), PG (1.13, 11.3, 22.6 μg/ml) and CG (1.05, 10.5, 21.0 μg/ml) and extracted (n=6) as part of one analytical run. To determine the between-day variabilities of the assays two quality control samples were extracted in triplicate with each analytical run (n=5). These quality control samples were 8 h urine samples collected following the ingestion of a 30 mg dose of DM and a 100 mg dose of PG, from an individual who was a PM of proguanil and an EM of dextromethorphan and an individual who was an EM of proguanil and a PM of dextromethorphan. To determine the accuracy of the assay ten urine samples, to which 0.5–10 μg/ml DR and DM and 1–20 μg/ml CG and PG were added, were analysed. Using linear regression analysis the concentrations of the analytes measured in each sample were compared with the concentrations added. The limit of detection of each analyte was defined as the smallest amount injected on column which resulted in a peak height four times greater than the baseline noise (S/N=4).

2.8. Dextromethorphan and proguanil phenotyping

The Human Research Ethics Committee of Royal North Shore Hospital (Sydney, Australia) approved this study (HREC Protocol No. 9502-027M(CTN)). Healthy, unrelated volunteers ingested a 30 mg capsule of dextromethorphan hydrobromide B.P. (81 μmol) (Pharmacy Department, Royal North Shore Hospital, Sydney, Australia) and a 100 mg proguanil hydrochloride tablet (Paludrine®, ICI Pharmaceuticals, Macclesfield, UK) (345 μmol) with 200 ml of water. All urine voided for the following 8 h was collected. The volume of urine and its pH were measured and recorded and aliquots of urine were stored at −20°C pending analysis.

3. Results and discussion

Representative chromatograms of DM and DR are shown in Fig. 2 and of PG and CG are shown in Fig. 3. Other HPLC techniques for measuring the con-

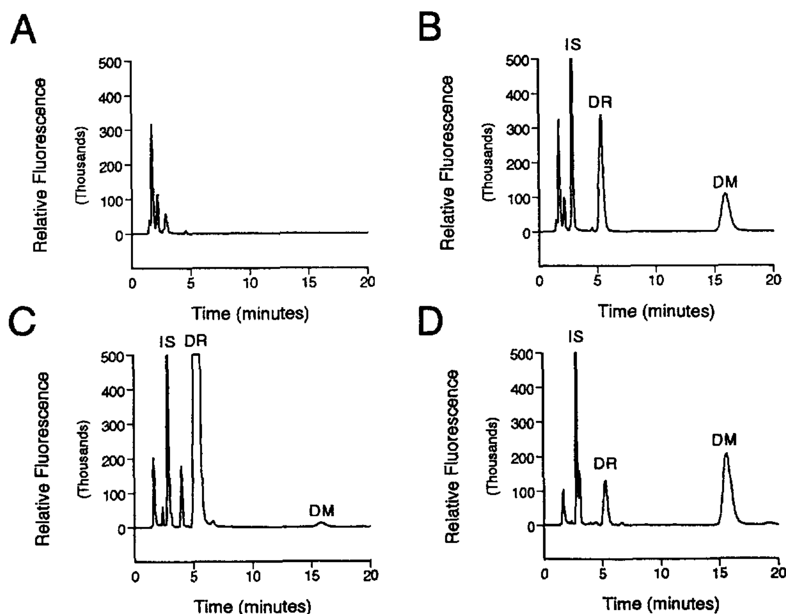

Fig. 2. Representative chromatograms of dextromethorphan (DM) and dextrorphan (DR) detected by native fluorescence. (A) Drug-free urine and (B) drug-free urine with DM (1.0 μg/ml) and DR (1.1 μg/ml) and pholcodine (I.S., 25 μg/ml) added. Chromatograms (C) and (D) are 0–8 h post-dose urine samples from healthy volunteers after 30 mg DM and 100 mg PG. (C) EM CYP2D6/PM CYP2C19 (DM 0.1 μg/ml, DR 7.8 μg/ml) and (D) PM CYP2D6/EM CYP2C19 (DM 0.5 μg/ml, DR 0.4 μg/ml).

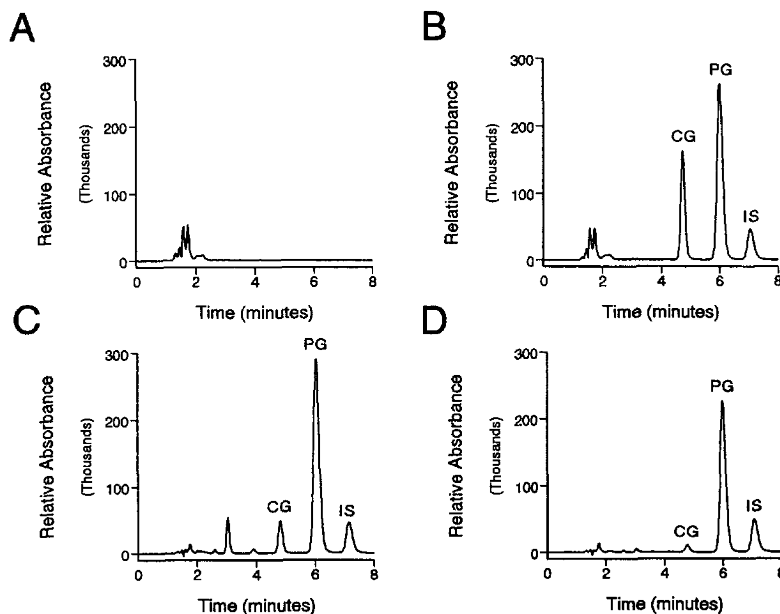

Fig. 3. Representative chromatograms of proguanil (PG) and cycloguanil (CG). (A) Drug-free urine and (B) drug-free urine with PG (15.8 μg/ml) and CG (17.0 μg/ml) and chlorcycloguanil (I.S., 4 μg/ml) added. Chromatograms (C) and (D) are 0–8 h post-dose urine samples from healthy volunteers after 30 mg DM and 100 mg PG. (C) EM CYP2C19/PM CYP2D6 (PG 13.6 μg/ml, CG 5.2 μg/ml) and (D) PM CYP2C19/EM CYP2D6 (PG 10.1 μg/ml, CG 0.8 μg/ml).

centrations of PG and CG in biological fluids have used UV absorption at 238 nm or 254 nm [14–16]. The detector wavelength of 238 nm was selected for this assay because it corresponds with the maximal UV absorbance for CG [14]. At 238 nm, DM and its metabolites can be detected (although the sensitivity is low) however using the mobile phase described by Chen et al. [10] PG, CG and two metabolites of DM, DR and 3-hydroxymorphinan, were not resolved. In order to optimise the separation of 3-hydroxymorphinan and DR from PG and CG, the mobile phase was modified from acetonitrile–triethylamine–double distilled water 17:0.06:82.94 to 6:0.12:93.88 (v/v/v) maintaining a pH of 3. The resulting retention times of pholcodine, 3-hydroxymorphinan, DR, 3-methoxymorphinan and DM (detected by native fluorescence) were 2.8, 3.9, 5.3, 11.2, and 15.8 min and the retention times of CG, PG and internal standard, chlorcycloguanil (detected by UV absorption) were 4.7, 6.0 and 7.0 min. The sample run-time was set at 20 min to allow for late eluting peaks in the fluorescence chromatograms. A small endogenous peak not resolved from pholcodine was swamped by adding a relatively high amount of the internal standard and did not compromise the between subject variability of the DM and DR assays.

Urine was extracted using the technique described by Chen et al. [10]. DR is eliminated principally as the glucuronide conjugate and therefore β-glucuronidase pretreatment was required prior to extraction. PG and CG could not be measured in the urine samples following β-glucuronidase treatment because the increased ratio of the aqueous phase to organic phase decreased the recovery of both CG and the internal standard, chlorcycloguanil. Consequently, the concentrations of PG and CG were measured in separate aliquots of urine which were not pretreated. Table 1 lists the recoveries of all analytes measured. The extraction efficiency was 70% for DR and 31% for DM over a concentration range of 0.5–10 μg/ml and 45% for CG and 100% for PG over a concentration range of 1–20 μg/ml.

The within-day and between-day reproducibilities for all four analytes expressed as coefficients of

Table 1
The recoveries of the analytes measured at three different concentrations

Concentration added (μg/ml)	n	Recovery (%, mean)	C.V. (%)
Dextrorphan			
0.54	6	70.0	4.0
5.4	6	71.7	3.6
10.8	6	68.3	3.6
Dextromethorphan			
0.49	6	32.4	3.8
4.9	6	30.7	4.1
9.7	6	29.6	3.2
Cycloguanil			
1.13	6	46.1	6.1
11.3	6	46.0	3.4
22.6	6	42.8	3.4
Proguanil			
1.05	6	99.1	5.3
10.5	6	100.9	1.6
21.0	6	106.6	2.4

Results are expressed as a percent of analyte added (mean and percent coefficient of variation, C.V.)

variation (C.V.) are presented in Tables 2 and 3. The within and between assay C.V. values were less than 14% for PG and CG and 17% for the lowest concentrations of DR and DM measured (DR 0.3 μg/ml, DM 0.1 μg/ml). The limits of detection of the assays were 0.1 μg/ml for DR and DM, 0.2 μg/ml for CG and 0.1 μg/ml for PG. Other HPLC techniques report greater sensitivity for PG and CG [14,15], however, in all 85 samples assayed to date the concentrations of PG and CG in urine have been at least 2.5 and 0.4 μg/ml, respectively indicating that the sensitivity of the present technique is adequate for the purposes of PG phenotyping. If greater sensitivity of the PG/CG assay were required, larger volumes of the sample extract (>5 μl) could be injected on column. A small peak at 3.8 min was apparent in the urine of extensive but not poor metabolisers of proguanil (Fig. 3). This peak may correspond to the minor metabolite of PG, 4-chlorophenylbiguanide whose formation may also be catalysed by CYP2C19 [17,18]. Very rapid EMs of DM had low urinary DM concentrations (<0.3 μg/ml) and consequently for these subjects larger aliquots of the extracted samples (15 μl) were reinjected onto the HPLC column.

Table 2
Within-day reproducibilities of the measurement of dextrorphan, dextromethorphan, cycloguanil and proguanil in urine

Concentration added (μg/ml)	n	Concentration measured (μg/ml) (mean±S.D.)	C.V. (%)
Dextrorphan			
0.54	6	0.53±0.04	6.7
5.4	6	5.4±0.3	4.5
10.8	6	11.4±0.6	5.1
Dextromethorphan			
0.49	6	0.48±0.04	8.1
4.9	6	4.9±0.3	5.0
9.7	6	10.2±0.4	4.0
Cycloguanil			
1.13	6	0.85±0.06	6.6
11.3	6	12.2±0.2	1.2
22.6	6	24.3±0.7	2.8
Proguanil			
1.05	6	1.24±0.03	2.3
10.5	6	10.8±0.3	3.0
21.0	6	21.8±0.6	2.7

Table 3
Between-day reproducibilities of the measurement of dextrorphan, dextromethorphan, cycloguanil and proguanil in 0–8 h urine from an EM CYP2D6/PM CYP2C19 and a PM CYP2D6/EM CYP2C19

	n	Concentration measured (μg/ml) (mean±S.D.)	C.V. (%)
Dextrorphan			
PM CYP2D6	6	0.36±0.02	4.3
EM CYP2D6	6	7.8±0.3	3.5
Dextromethorphan			
PM CYP2D6	6	0.54±0.04	7.0
EM CYP2D6	6	0.12±0.02	16.7
Cycloguanil			
PM CYP2C19	6	0.8±0.1	13.8
EM CYP2C19	6	5.2±0.2	4.0
Proguanil			
PM CYP2C19	6	10.4±0.5	4.4
EM CYP2C19	6	13.6±1.0	7.1

The relationships between peak height ratios and urinary concentrations were linear for all analytes over the concentration ranges studied. Typical standard curves were $y=0.23x+0.02$ ($r^2=0.998$) for DM, $y=0.64x+0.01$ ($r^2=0.998$) for DR, $y=0.41x+0.05$ ($r^2=0.998$) for PG and $y=0.19x+0.00$ ($r^2=0.999$) for CG. In our system the DR peak height

ratio response was not linear for urinary concentrations of dextrorphan greater than 20 μg/ml when 5 μl of the extracted sample was injected on column. To measure concentrations of DR greater than 20 μg/ml smaller aliquots (2 μl) of extracted samples were reinjected. Caffeine and its metabolites did not interfere with the detection of any of the analytes, therefore this HPLC technique is also suitable for the identification of CYP2D6 and CYP2C19 activity when caffeine has been used to assess N-acetyltransferase-2 activity. The accuracy of each assay was good. The relationships between the concentrations of the analytes added and the measured concentrations ($n=10$) were $y=0.97x+0.01$ ($r^2=0.998$) for DM, $y=0.93x+0.03$ ($r^2=0.999$) for DR, $y=1.10x+0.40$ ($r^2=0.992$) for PG and $y=0.90x+0.27$ ($r^2=0.996$) for CG.

In summary, a novel technique to measure PG and CG in urine which avoids the use of expensive ion-pairing agents and/or solid-phase extraction columns, is described. The method can also be used to measure DM and DR in urine. The two drugs and their polymorphic metabolites can therefore be measured in urine using a common extraction procedure and a single HPLC system. If desired all four analytes can be measured in a single analytical run. For laboratories currently measuring both pharmacogenetic probe drugs using separate assays, adopting this technique will reduce the HPLC equipment, reagents and staff time required to analyse samples following phenotyping for the genetic polymorphisms in cytochromes P450 2D6 and 2C19.

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