

## HPLC Measurement, Blood Distribution, and Pharmacokinetics of Oral Clotrimazole, Potentially Useful Antisickling Agent

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Clotrimazole (CLT) has recently been shown to be a potent and specific inhibitor of the Ca<sup>2+</sup>-activated K<sup>+</sup> channel and to thereby prevent K<sup>+</sup> loss and cellular dehydration of sickled erythrocytes. This evidence suggests that oral CLT may be a useful new therapy for sickle cell disease. Here, we describe the development of an HPLC assay to measure CLT, a method we used to study the pharmacokinetics and transport of CLT in normal volunteers. The assay's linear range extended to 10  $\mu\text{mol/L}$ ; the detection limit was 0.1  $\mu\text{mol/L}$ , analytical recovery 97.7%, and run-to-run imprecision (CV) <4.7%. In unaffected subjects, CLT concentration peaked within 6 h of oral administration and returned to close to baseline by 24 h. High-density lipoproteins appear to be the main carriers of this drug in both normo- and hypertriglyceridemic plasma. We conclude that the method described here is ideally suited for therapeutic monitoring of CLT concentrations.

**Indexing Terms:** drug assays/sickle cell disease/hemoglobinopathies/ion-transport channels/calcium/potassium/lipoproteins/hypertriglyceridemia/Gardos channel/chromatography, reversed-phase

Clotrimazole (CLT) is a highly effective drug against a broad spectrum of fungi (1, 2).<sup>7</sup> In addition to its antimycotic activity, CLT has been shown in vitro to specifically and potently inhibit the Ca<sup>2+</sup>-activated K<sup>+</sup> channel, known as the Gardos channel, in sickle erythrocytes (3, 4). The inhibition of this channel prevents K<sup>+</sup> loss and cellular dehydration. Furthermore, as recently shown, oral treatment with CLT inhibits the Gardos channel, increases cellular water and K<sup>+</sup> content, and decreases cell density and mean corpuscular hemoglobin concentration in a transgenic mouse model of human sickle cell disease (4). This evidence suggests that oral administration of CLT may become an important new prototype of antisickling agents that prevent the dehydration of erythrocytes. A clinical trial of CLT in sickle cell disease is already in progress, and reliable determination of blood or plasma CLT concentration is an essential step in monitoring and optimizing this therapy.

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<sup>7</sup> Nonstandard abbreviations: CLT, clotrimazole; HDL, high-, LDL, VLDL, low-, and very-low-density lipoprotein, respectively; TG, triglyceride; AUC, area under the curve.

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Four assays have already been described. Two HPLC methods determine CLT in pharmaceutical formulations (5, 6). One bioassay and one two-dimensional paper chromatographic method measure CLT in various body fluids (7, 8). Here, we describe the development of a reversed-phase HPLC assay for determining CLT concentration in whole blood and plasma. We used the assay to study the pharmacokinetics and distribution of CLT in the blood.

### Materials and Methods

#### Reagents

Clotrimazole and the internal standard, testosterone propionate, were obtained from Sigma Chemical Co., St. Louis, MO. 2-Chlorophenyl-bis-phenyl-methanol, a CLT metabolite, was a gift from Miles Inc., West Haven, CT. HPLC-grade acetonitrile, ethanol, and methanol, and dibasic potassium phosphate were purchased from Fisher Scientific, Fair Lawn, NJ. Various drugs that we tested for potential interference in the assay were obtained from Sigma Chemical Co.; Aldrich Chemical Co., Milwaukee, WI; Roche Diagnostic Systems, Branchburg, NJ; U.S. Pharmacopeial Convention, Rockville, MD; Pfizer Laboratories Div., New York, NY; ICN Biochemicals, Cleveland, OH; and Pfaltz & Bauer, Stamford, CT.

Enzymatic reagents for the determination of cholesterol and triglycerides (performed with correction for endogenous glycerol) were obtained from Boehringer Mannheim, Indianapolis, IN. Dextran sulfate (50 000 Da) for the isolation of high-density lipoprotein (HDL) fraction was from Genzyme, Cambridge, MA.

Calibrators, at concentrations of 0.5, 1.0, 2.0, and 5.0  $\mu\text{mol/L}$ , and controls, at concentrations of 1.0 and 5.0  $\mu\text{mol/L}$ , were prepared by mixing filtered plasma with methanol-based stock CLT solution (100  $\mu\text{mol/L}$ ). The calibrators, controls, and methanol-based internal standard solutions (100  $\mu\text{mol/L}$ ) were aliquoted and stored at  $-70^{\circ}\text{C}$  until use. The mobile-phase mixture was prepared by combining 275 mL of dibasic potassium phosphate (25 mmol/L, adjusted to pH 6.3 with 0.67 mol/L phosphoric acid) and 725 mL of methanol. The mobile phase is stable in amber glass at room temperature for at least 1 month.

#### Apparatus

We used a Model LC-10A liquid chromatograph, SCL-10A liquid controller, SPD-10A UV/Vis detector, SIL-10A autoinjector, and CR-501 Chromatopac integrator/recorder (all from Shimadzu Scientific Instru-

ments, Columbia, MD) and a 5 cm × 4.6 mm column [Supelcosil packing (LC-8DB), 5- $\mu$ m particles size, from Supelco, Bellefonte, PA]. Bond-Elut disposable reversed-phase (C<sub>18</sub>) extraction columns (100 mg bed-size) and a VacElut vacuum manifold, which was used in the extraction procedure according to the manufacturer's recommendations, were obtained from Analytichem International, Harbor City, CA. We also used a TurboVac evaporator (Zymark Corp., Hopkinton, MA) in this procedure.

For lipoprotein fractionation we used the fixed-angle rotor TLA-100 and the TL-100 tabletop ultracentrifuge (Beckman Instruments, Palo Alto, CA). The cellulose propionate tubes were sliced with a CentriTube slicer (Beckman Instruments). Cholesterol and triglyceride were measured with the Hitachi 911 analyzer (Boehringer Mannheim) according to the manufacturer's recommendation. Our laboratory is enrolled in the Lipid Standardization Program of the National Heart, Lung, and Blood Institute/Centers for Disease Control and Prevention.

#### Procedures

**Chromatographic determination of CLT.** We added 50  $\mu$ L of the internal standard solution and 3 mL of 150 mL/L acetonitrile to 200  $\mu$ L of a patient's plasma or whole blood, calibrators, and controls and vortex-mixed for 30 s. We then applied the mixtures to the C<sub>18</sub> columns, which we had preconditioned by sequential additions of 2 mL of 950 mL/L ethanol and 2 mL of 150 mL/L acetonitrile. Columns were then washed with 9 mL of 300 mL/L acetonitrile and, after drying, eluted with 200  $\mu$ L of 950 mL/L ethanol. We collected the eluates and injected 10  $\mu$ L of each into the HPLC, with the same mobile-phase mixture as described above. The chromatographic separation was performed at ambient temperature with a flow rate of 1.0 mL/min; the absorbance of the effluent was monitored at 210 nm.

**Separation of free from protein-bound CLT.** Free CLT was separated from the protein-bound fraction by centrifuging 500  $\mu$ L of plasma in a Centrifree Micropartition System filter (Amicon Division, W. R. Grace & Co., Beverly, MA) at 1500g for 15 min at 4°C. CLT concentrations were determined in the original specimen and in the ultrafiltrate, which was considered to contain the free fraction. Albumin-bound and free CLT were separated from the other protein-bound fraction using a 100 000-Da-cutoff filter (Centricon-100 Concentrator; Amicon Division). After centrifuging 500  $\mu$ L of plasma at 1000g for 30 min at 4°C, we determined the CLT concentrations in the original specimen and in the filtrate, which represented the free and albumin-bound fractions.

**Lipoprotein separation and lipid quantification.** Lipoproteins were separated with the Lipid Research Clinics' beta-quantification assay (9). We centrifuged 230  $\mu$ L of plasma obtained 6 h after the CLT dose at 415 000g for 3 h. The sample-containing tubes were then sliced to separate the very-low-density lipoprotein (VLDL) from the low-density lipoprotein (LDL) and the

HDL fraction. The LDL fraction was then precipitated by dextran sulfate-Mg<sup>2+</sup> solution as previously described (10). The triglyceride and cholesterol content of each fraction was determined enzymatically. The CLT concentration was also determined in each of the lipoprotein fractions. In addition, we added CLT to normo- and hypertriglyceridemic plasma samples and incubated these, with shaking, in a waterbath at 37°C for 5 h. Lipoproteins were then separated and the cholesterol, triglyceride, and CLT concentrations determined in each lipoprotein fraction.

**Pharmacokinetic studies.** Four healthy adults (three men and one woman), ages 38 to 45 years, took 1000 mg of CLT orally immediately after a standardized meal. The average weight of the subjects was 68 kg (range 55.3–72.5). None was a smoker. Heparinized blood specimens were collected into heparin-containing vessels at 0, 2, 4, 6, 8, 24, 36, and 96 h postdose. In two subjects, urine was also collected at 4 h. The protocol of this study was approved by the Institutional Review Board of our institution, and appropriate consent forms were signed by the participants. Both whole-blood and plasma CLT concentrations were determined in all blood specimens. Basic pharmacokinetic variables, including area under the plasma concentration curve from time zero to infinity (AUC<sub>0- $\infty$</sub> ), elimination half-life, maximum drug plasma concentration, and apparent volume of distribution, were calculated by using the PCNONLIN program (ClinTrials Research, Lexington, KY).

**Statistical analysis.** Correlations between the various variables were calculated by least-squares regression analysis. Student's *t* probability test was used to determine the difference between the means of two populations, with *P* < 0.05 considered significant.

#### Results and Discussion

A typical chromatogram from the plasma or whole-blood extract of a subject taking CLT is presented in Fig. 1. Our method demonstrates two CLT metabolites. The absolute retention times of metabolite I, metabolite II,

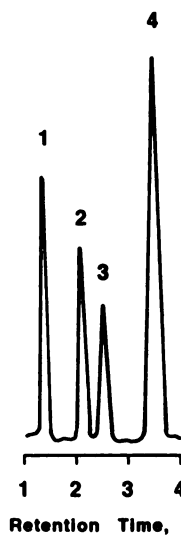


Fig. 1. Typical chromatogram of extracted CLT whole-blood or plasma specimen: (1) CLT metabolite I (1.55 min); (2) CLT metabolite II (2.15 min); (3) CLT (2.60); (4) the internal standard, testosterone propionate (3.60).

and CLT are 1.55, 2.15, and 2.60 min, respectively. Duhm et al. have previously reported the presence of two major and one minor metabolites in plasma after oral administration of CLT to rats (8). Their two main metabolites were identified as 2-chlorophenyl-4-*O*-hydroxyphenyl-*O*-phenylmethane and 2-chlorophenyl-bisphenylmethane; 2-chlorophenyl-bis-phenyl-methanol was a minor species. In our system, a pure preparation of 2-chlorophenyl-bis-phenyl-methanol had a retention time of 2.15 min, corresponding to metabolite II. The exact chemical structures of the two metabolites are currently being determined by gas chromatography-mass spectrometry. An extracted urine specimen shows the same chromatogram as in Fig. 1 but without the CLT peak (chromatogram not shown), in agreement with a previous report (8). We are currently testing the ability of these metabolites to inhibit the Gardos channel.

The calibration curves in both whole blood and plasma, with CLT concentrations ranging from 0 to 5  $\mu\text{mol/L}$ , were linear (for whole blood, slope = 0.82 and  $r = 0.99$ ; for plasma, slope = 1.28 and  $r = 0.99$ ) and reproducible: For whole blood, the average CV for the calibrators was 7.1% ( $n = 18$ ); for plasma, the average CV for the calibrators was 4.9% ( $n = 21$ ). Because of the significantly different slopes ( $P < 0.0025$ ) of the calibration curves of CLT in whole blood and plasma, the type of calibrators should be the same as the type of specimens for accurate determination of CLT concentration.

The day-to-day precision, reflected by CV, was determined with 10 replicates of plasma-based quality-control materials at two drug concentrations, with assay of a newly extracted set of calibrators each day. For drug concentrations of 1 and 5  $\mu\text{mol/L}$ , the precision was 4.7% and 2.6%, respectively. A recovery study was performed by adding to drug-free plasma and whole blood from four subjects known amounts of the purified drug. At CLT concentrations of 0.5, 1, and 2  $\mu\text{mol/L}$ , the mean (SD) analytical recoveries in plasma were 99.6% (6.7%), 97.2% (7.9%), and 96.3% (7.6%), and in whole blood were 90.4% (8.8%), 94.2% (7.0%), and 100% (4.8%), respectively. Furthermore, the assay could detect as little CLT as 0.1  $\mu\text{mol/L}$ , with a linear calibration curve up to 10  $\mu\text{mol/L}$ . These limits of analytical range more than encompass the expected therapeutic concentrations of CLT (0.2–5.0  $\mu\text{mol/L}$ ).

To further validate the accuracy of this assay, we conducted an interference study with 48 commonly prescribed drugs and some over-the-counter medications (Table 1). Of the drugs tested, only two were able to be extracted and detected in this assay: Itraconazole and doxepin eluted with retention times of 2.55 and 2.75 min, respectively. However, the retention time of only itraconazole was somewhat similar to that of CLT. We point out, however, that this assay can be assumed to be free of interference from several hundred other drugs that would not be coextracted under the conditions reported here or that would have significantly different retention times.

Seo et al. have reported that the CLT concentration determined by a bioassay is about threefold higher in

**Table 1. Drugs used in the interference study.**

<i>Antiepileptics</i>	<i>Antidepressants</i>	<i>Other drugs</i>
Barbituric acid	Amitriptyline	Acetaminophen
Carbamazepine	Desipramine	Acetylsalicylic acid
Carbamazepine epoxide	Desmethyldoxepin	Brompheniramine
Clonazepam	Doxepin (2.75) <sup>a</sup>	Caffeine
Ethosuximide	Imipramine	Chlorpheniramine
Felbamate	Maprotiline	Digitoxin
Mephentoin	Nortriptyline	Digoxin
Mephobarbital	Protriptyline	Ibuprofen
Metharbital	<i>Antibiotics</i>	Itraconazole (2.55) <sup>a</sup>
Methsuximide	Chloramphenicol	Phenylpropanolamine
Methylsuccinimide	Gentamicin	Theophylline
Paramethadione	Tobramycin	
Phenacemide	Vancomycin	
Phenobarbital	<i>Antiarrhythmics</i>	
Phensuximide	<i>N</i> -Acetylleprocaïnamide	
Phenytoin	Disopyramide	
Primidone	Lidocaine	
Trimethadione	Procainamide	
Valproic acid	Quinidine	

<sup>a</sup> Drugs coextracted with CLT (2.6) and metabolites I (1.55) and II (2.15) at the retention times (minutes) indicated in parentheses.

whole blood than in serum, and that plasma CLT is about twice that of serum (7). Our data indicate that whole blood and plasma CLT concentrations change proportionally, up to 10 h, after a single drug dose. In contrast to the earlier findings, only about 37% (SD 8.4%) of whole-blood CLT is present in the cellular compartment, the remaining portion being in the plasma (determined at four time points in three subjects). In addition, we noted no significant difference between serum and plasma CLT concentrations (plasma  $0.55 \pm 0.47 \mu\text{mol/L}$ , serum  $0.67 \pm 0.58 \mu\text{mol/L}$ ,  $n = 4$ ,  $P < 0.5$ ). The exact cause of the discrepancy between the findings of our study and that of Seo et al. is unknown, but is probably related to the fact that the latter is a bioassay.

In the present study, we examined the distribution of CLT in plasma by determining its concentration in the albumin- and lipoprotein-bound fractions. Free CLT (or unbound CLT) concentration was also assessed. Our data indicate that free CLT is present in plasma in very minute amounts. In fact, its concentration was  $< 0.1 \mu\text{mol/L}$ , the detection limit of this assay. This finding is consistent with a previous report indicating that  $< 1\%$  of CLT is present in its active form (8). In addition, only  $\sim 4\%$  (SD 3.5%) of this drug was shown to be bound to albumin when determined in four subjects 4 h after dose.

Lipoproteins play a major role in the *in vivo* transport of many lipophilic drugs, and in part explain some of the variability observed in pharmacokinetic and pharmacodynamic profiles. Plasma cyclosporine of normotriglyceridemic subjects, for example, is transported mainly by HDL (11, 12); LDL and VLDL are also involved in the transport, but to a lesser extent. In

**Table 2. Distribution of CLT among the major lipoprotein classes of normo- and hypertriglyceridemic subjects<sup>a</sup>.**

	Mean $\pm$ SD		
	Lipid conc, mmol/L <sup>b</sup>	CLT in each lipoprotein, $\mu$ mol/L	Ratio of CLT:lipid mmol/mol
<i>Hypertriglyceridemic (n = 4)</i>			
VLDL	3.66 $\pm$ 0.56	0.23 $\pm$ 0.05	0.07 $\pm$ 0.02
LDL	5.56 $\pm$ 1.60	0.58 $\pm$ 0.10	0.11 $\pm$ 0.04
HDL	1.49 $\pm$ 0.81	1.08 $\pm$ 0.05	0.89 $\pm$ 0.47
<i>Normotriglyceridemic (n = 4)</i>			
VLDL	1.47 $\pm$ 0.73	0.13 $\pm$ 0.05	0.12 $\pm$ 0.09
LDL	5.01 $\pm$ 0.69	0.13 $\pm$ 0.60	0.03 $\pm$ 0.01
HDL	1.45 $\pm$ 0.37	0.53 $\pm$ 0.31	0.40 $\pm$ 0.30

<sup>a</sup> Mean  $\pm$  SD triglycerides in the normotriglyceridemic group is 1220  $\pm$  470 mg/L; in the hypertriglyceridemic group, 3920  $\pm$  290 mg/L.

<sup>b</sup> Represents cholesterol and triglyceride concentrations.

contrast, the majority of plasma cyclosporine in hypertriglyceridemic patients is carried by VLDL. We investigated the distribution of CLT in the lipoprotein fractions of four normotriglyceridemic subjects (mean  $\pm$  SD, 1220  $\pm$  470 mg/L) who received the drug orally, finding ~70% of CLT present in the HDL fraction, with the remaining drug almost equally distributed between LDL and VLDL (Table 2). Furthermore, when calculated on the basis of lipid content (cholesterol and triglyceride), HDL was the best lipoprotein carrier of CLT, and LDL was the poorest. When CLT was added in vitro to normotriglyceridemic plasma samples and incubated for 5 h at 37°C, the distribution of CLT in lipoprotein fractions was identical to that seen in the in vivo samples (data not shown). Therefore, we added CLT to hypertriglyceridemic plasma samples (mean  $\pm$  SD, 3920  $\pm$  290 mg/L; n = 4) to determine whether its distribution among the various lipoprotein classes was

affected by the triglyceride content. Our data indicate that, unlike cyclosporine and FK-506 (12, 13), the distribution of CLT among the lipoprotein classes in hypertriglyceridemic samples is similar to that seen in normotriglyceridemic samples, with HDL being the major lipoprotein carrier of CLT (80%) (Table 2). Compared with the other lipoproteins, HDL contains the lowest total lipid concentration but the highest amount of phospholipids per particle (14). These data suggest that phospholipids may constitute the actual binding sites and carriers of CLT.

The pharmacokinetics of a single oral dose (1000 mg), performed in four subjects, showed significant interindividual variation in the concentrations of CLT and its metabolites (Fig. 2). The peak concentration of CLT ranged from 0.3 to 3.3  $\mu$ mol/L, but always occurred within 4 h of dosing; the metabolites peaked within 6 h. The pattern of concentration changes for metabolites I and II differed considerably among the subjects. Subject B exhibited a different pattern from that of the other three by having CLT concentrations lower than those of the metabolites. These findings demonstrate marked interindividual variation in the absorption of the parent drug and of the metabolites, formed by the acid hydrolysis of CLT in the stomach. The elimination of CLT best fit a single-compartment model with the following parameters (mean  $\pm$  SE): volume of distribution 342.6  $\pm$  106.1 L, half-life 2.99  $\pm$  0.68 h, projected AUC<sub>0- $\infty$</sub>  12.59  $\pm$  1.14  $\mu$ mol/L  $\cdot$  h, K<sub>10</sub> 0.232  $\pm$  0.053, and maximum drug plasma concentration 2.33  $\pm$  0.61  $\mu$ mol/L. The correlation coefficient between the observed and the predicted concentrations using one compartment model was 0.97.

In conclusion, this chromatographic assay for the determination of CLT concentration is accurate, pre-

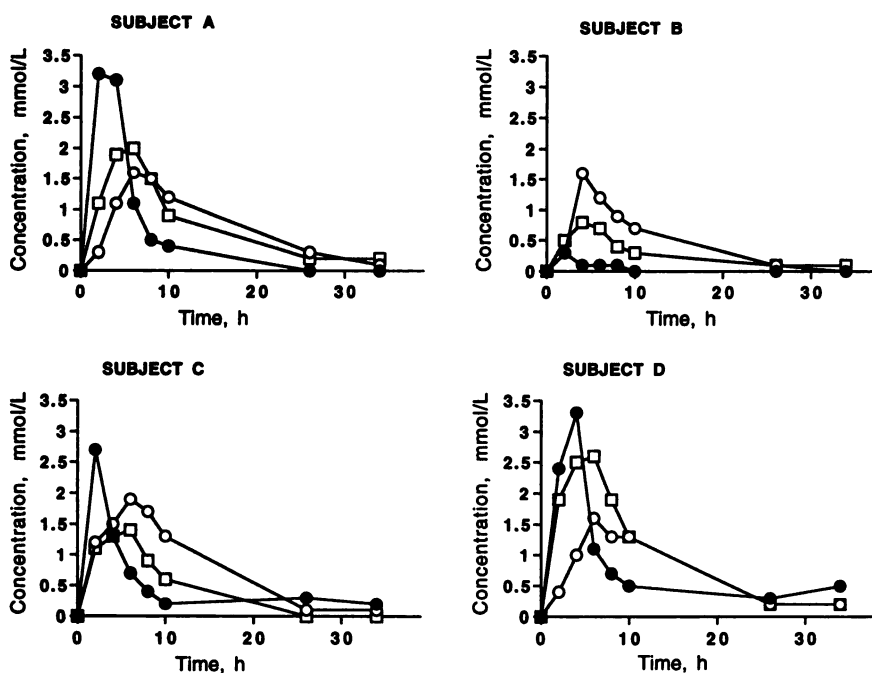


Fig. 2. Changes in CLT and metabolite concentrations in plasma after a single oral dose (1000 mg) in four subjects (A-D). ●, CLT concentrations; □, metabolite I concentrations; and ○, metabolite II concentrations.

cise, and sensitive and is ideally suited for the therapeutic monitoring of this drug. HDL appears to be the major lipoprotein carrier of this drug in plasma.

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