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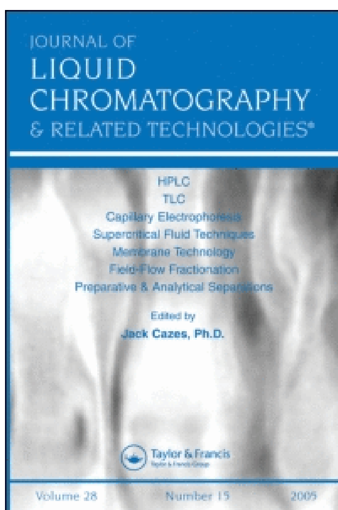

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Simultaneous Determination of Chloroquine and Desethylchloroquine in Blood, Plasma and Urine by High-Performance Liquid Chromatography

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SIMULTANEOUS DETERMINATION OF CHLOROQUINE AND DESETHYLCHLOROQUINE IN BLOOD, PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-performance liquid chromatographic method is described for the determination of chloroquine and its major metabolite desethylchloroquine in blood, plasma and urine. The procedure employs reversed-phase chromatography, with ultraviolet detection, and chlorpheniramine as an internal standard. One milliliter samples of biologic fluid are extracted in a single step with ether. The method has a sensitivity limit of 5 ng/ml for chloroquine and its metabolite. The applicability of the method is demonstrated by the analysis of blood and plasma samples obtained from rabbits following intravenous administration of chloroquine.

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INTRODUCTION

Chloroquine is the most effective drug employed in the treatment of malaria, a disease that affects approximately twenty percent of the world population. Chloroquine is also used in the treatment of systemic lupus, intestinal amebiasis and rheumatoid arthritis. Recently, several high performance liquid chromatographic methods have been reported (1-3) for the determination of chloroquine in biologic fluids. Two of these methods required fluorescence detection (1,2). The other method (3) employed ultraviolet detection, but did not separate the parent drug from its major metabolite, desethylchloroquine (4). Further, two of the assays (1,3) utilized internal standards which had to be synthesized.

The present study concerns the development of a simple one-step extraction method, employing a readily available drug, chlorpheniramine, as an internal standard. The reversed-phase HPLC procedure utilizes ultraviolet detection, and permits the simultaneous assay of chloroquine and desethylchloroquine in plasma, whole blood and urine.

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EXPERIMENTAL

Reagents and Materials

Chloroquine diphosphate was obtained from Sigma Chemical Co. (St. Louis, MO); desethylchloroquine was provided by Sterling-Winthrop Research Institute (Rensselaer, NY); and chlorpheniramine

maleate was obtained from Berlex Laboratories, Inc. (Cedar Knolls, NJ). Methanol and chloroform were HPLC-grade, Fisher Scientific (Fair Lawn, NJ). Deionized water was used throughout the experiment and all other chemicals were of reagent grade.

Glass tubes employed in the extraction and evaporation procedures were placed in a 500° kiln for 1 hr to eliminate any contaminants remaining after washing with detergent, rinsing with deionized water and drying at 100° overnight. The 15 ml conical tubes used for evaporation were silanized with 4% trimethylchlorosilane from Alfa Products (Danvers, MA) in dry toluene. After silanization, the tubes were rinsed with methanol and dried at 100° overnight.

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Chromatography Equipment and Conditions

The HPLC system (Waters Assoc., Milford, MA) consisted of a Model M6000 solvent delivery system, a U6K loop injector and a prepacked 30 cm x 3.9 mm i.d. stainless steel column containing 10 μm C_{18} μ-Bondapak, along with a 23mm x 3.9mm guard column, hand packed with C_{18}/Corasil. The variable wavelength detector, Kratos Analytical Instruments (Westwood, NJ), was set at 225 nm, 0.02 aufs.

The mobile phase, consisting of 0.18% ammonium hydroxide (30%) in methanol, was deaerated by filtering through a type HA filter, pore size 0.45 μm, using a solvent clarification kit (Millipore, Bedford, MA). The flow rate was 2 ml/min, and the system was operated at ambient temperature.

Standard Solutions

Aqueous solutions were prepared containing 5 μg/ml of chloroquine diphosphate and desethylchloroquine. Aliquots of this solution were diluted with deionized water to provide standard solutions for the calibration curves ranging from 25 ng/ml to 2.0 μg/ml of drug and metabolite. The aqueous internal standard solution contained 0.5 μg/ml of chlorpheniramine maleate.

Standard Curves and Biologic Samples

Standard curves were prepared with pooled human or rabbit plasma and blood, and human urine. One milliliter of blood, plasma or urine was combined with 1 ml of chloroquine/desethylchloroquine standard solution, 1 ml of 0.5 μg/ml internal standard solution, and 1 ml of 1.0 N sodium hydroxide in a 20-ml polytef-lined screw-cap centrifuge tube. The mixture was extracted with 8 ml of chloroform for 20 min, with gentle mixing on a platform shaker. After centrifugation at -10° for 15 min at 3000 rpm, the aqueous layer was aspirated off and the chloroform layer was transferred to 15 ml conical tubes and evaporated under a stream of nitrogen at 40°. The residue was reconstituted with 100 μl of methanol and vortexed for 20 sec. A 10 μl aliquot was then injected into the HPLC. An unweighted least-squares regression was employed to fit plots of peak height ratio (drug or metabolite/internal standard) versus drug or metabolite concentration.

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Studies in Rabbits

Chloroquine diphosphate was administered intravenously at a dose of 10 mg/kg (equivalent to 6.2 mg/kg base) through the marginal ear vein of New Zealand White rabbits. Five milliliter blood samples were withdrawn over an 8 hr period through an arterial catheter, and were placed into tubes containing 50 U of heparin. A 2 ml aliquot of whole blood was frozen, and a 3 ml aliquot was centrifuged to obtain the plasma, which was also frozen until the time of assay.

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RESULTS AND DISCUSSION

Figure 1 illustrates typical chromatograms obtained for the assay of human urine, blood and plasma containing 0.5 μg/ml of chloroquine diphosphate and desethylchloroquine, as well as 0.5 μg/ml of internal standard. The retention times for the internal standard, drug and metabolite were 2, 4 and 8 min, respectively. Blank samples of each biologic fluid did not exhibit any interfering peaks in the chromatograms. The recovery of drug and metabolite from urine, plasma and blood samples containing 0.5 μg/ml of each component was consistently in the range of 85-87 percent. The recovery of internal standard averaged 90-95 percent from urine, plasma and blood. The precision of the assay was evaluated with ten replicate urine, plasma and blood samples containing 0.05 μg/ml of drug and metabolite, and ten samples of each biologic fluid containing 0.15 μg/ml of drug and metabolite.

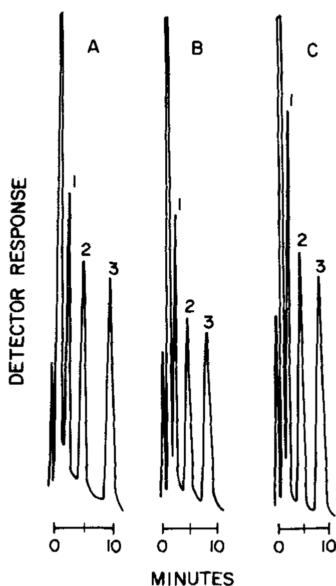

Figure 1 - Chromatograms from the assay of human urine (A), blood (B) and plasma (C) containing 0.5 µg/ml of internal standard (1), chloroquine (2) and metabolite (3).

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The relative standard deviations (SD x 100/mean) were 8.7 percent and 4.9 percent for the lower and higher concentrations, respectively. Standard curves for drug and metabolite in each biologic fluid exhibited excellent linearity over a concentration range of 25 ng/ml to 2.0 µg/ml, with coefficients of determinations (r^2) which were consistently at least 0.998. The intercepts did not differ significantly from zero. The limit of detection of both chloroquine and its metabolite is 5 ng/ml of biologic fluid.

Figure 2 is representative of chromatograms obtained by assaying blood and plasma samples obtained from rabbits receiving

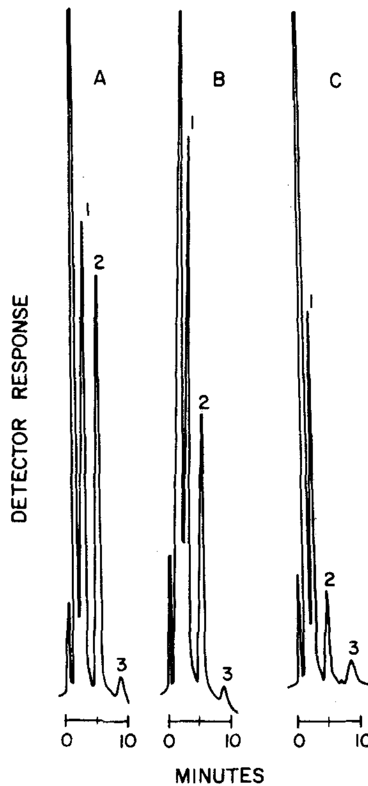

Figure 2 - Chromatograms for the assay of rabbit blood and plasma following administration of 6.2 mg/kg of chloroquine base intravenously. Key: (A), 15 min blood; (B), 30 min blood; (C), 30 min plasma; 1, internal standard; 2, chloroquine; 3, metabolite.

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an intravenous dose of chloroquine. Two features of these chromatograms are of particular interest. First, the metabolite is well separated from the chloroquine, and is present in blood and plasma obtained within 15 min after an intravenous dose. In subsequent studies to be reported elsewhere, desethylchloroquine was detected in blood samples obtained as early as 5 min after dosing,

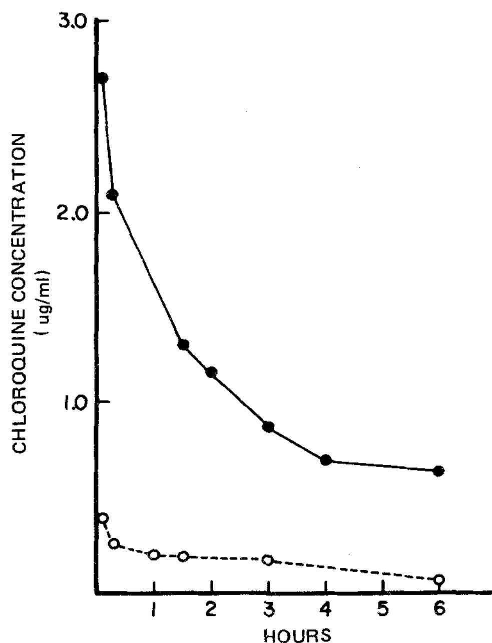

Figure 3 - Blood (●) and plasma (○) concentration-time profile for a rabbit receiving a 6.2 mg/kg intravenous dose of chloroquine.

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and the metabolite concentrations remained relatively constant, ranging from 50-100 µg/ml, for 24 hr after dosing. Secondly, the data shown in Figure 2 indicate that chloroquine concentrations in whole blood samples are considerably higher than those found in corresponding plasma samples. This observation is consistent with previous work (5). Figure 3 represents blood and plasma chloroquine concentrations determined in another rabbit following a 6.2 mg/kg intravenous dose. The blood concentrations are approximately seven-fold greater than those found in plasma.

Previous studies in humans have indicated peak plasma chloroquine concentrations of approximately 80 ng/ml and 1 μg/ml following single dose administration of 300 mg of chloroquine base by oral and intravenous routes, respectively (1). Further, during chronic daily administration of 300 mg of chloroquine base, blood chloroquine concentrations ranged from approximately 150-500 ng/ml (3). Concentrations of chloroquine in the urine of these patients ranged from 17-20 μg/ml. Therefore the present assay, which is relatively rapid and is specific for chloroquine and its major metabolite, is applicable to pharmacokinetic studies in animals and man, as well as suitable for routine monitoring of patients who are receiving this drug.

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