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Using a Coupled Achiral-Chiral HPLC System to Determine the Mefloquine Enantiomers in Plasma and Whole Blood.

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Introduction.

The stereoisomeric composition of drug substances is rapidly becoming a key issue in the development, regulation and administration of pharmaceuticals. This concern has centered on the enantiomeric composition of these substances, and has been spurred on by the recognition that the enantiomers of a pharmacologically active compound often differ in their efficacy, toxicity and pharmacological disposition.

The most widely used analytical technique for the separation and quantitation of enantiomers is HPLC. The separation of enantiomers is a significant HPLC challenge because the physical and chemical properties of the two optical isomers are identical. Typical chiral analysis approaches include the use of specialty chiral columns, chiral mobile phase modifiers and derivatization using homochiral reagents. When enantiomers must also be resolved from other non-chiral compounds the HPLC analysis becomes even more difficult. To solve this difficult problem a coupled achiral-chiral HPLC system has been developed. This technique has been used to determine the mefloquine (MFQ) enantiomers in plasma and whole blood.

Mefloquine is a chiral molecule which is used for the treatment and prophylaxis of malaria. The agent is administered orally as a racemic mixture of the (+) and (−) forms, Figure 1. Initial studies of MFQ pharmacokinetics have demonstrated that the drug has a long half-life, but these studies did not consider the plasma disposition and elimination of the MFQ enantiomers. The potential importance of determining the pharmacological fate of the separate MFQ enantiomers was indicated by a study which found that (−) MFQ is a more potent acetylcholinesterase and butylcholinesterase inhibitor than (+) MFQ[1]. In addition, there have been conflicting studies regarding differential accumulation of MFQ in erythrocytes versus plasma[2, 3]. The enantioselectivity of this accumulation had not been studied.

The initial separation (non-enantiomeric separation) was performed on an achiral column. The total quantity of (+) and (−) MFQ was determined on the cyano bonded phase column and any interfering components in the biological matrix were removed during this first separation. The enantiomeric composition [(+) and (−) MFQ ratio] was determined on the (S)-naphthylurea chiral stationary phase. The two columns were connected by a switching valve equipped with a silica precolumn. The precolumn concentrated the MFQ in the eluent from the achiral column before backflushing onto the chiral phase. Although this coupled column system was specifically validated and applied to the pharmacokinetic analysis pilot study for the (+) and (−) MFQ concentration in plasma and whole blood, the technique can readily be applied for many other chiral compounds where matrix affects are a major concern and sensitivity is a major issue.

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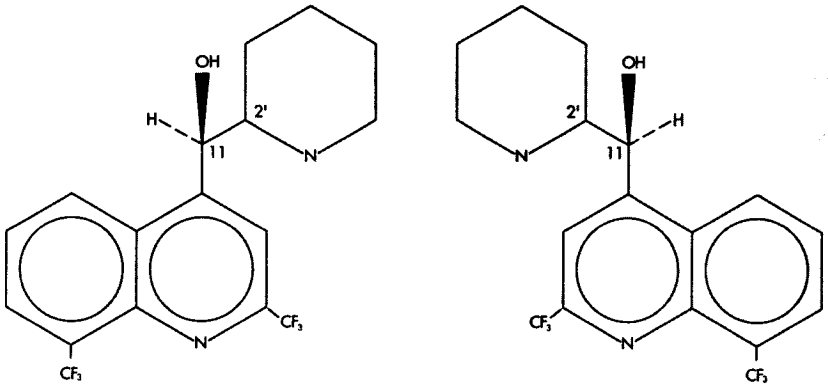
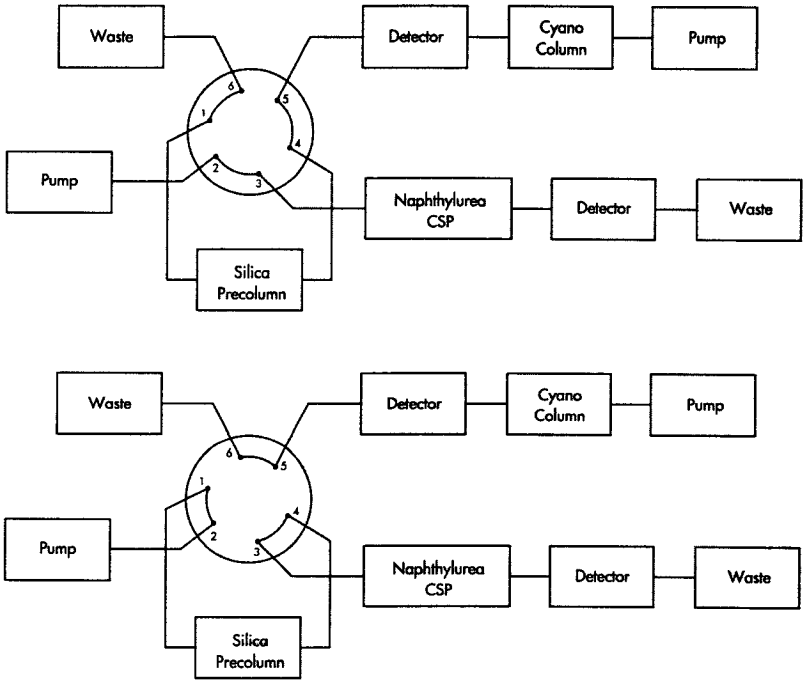
Figure 1: Structure of Mefloquine Enantiomers.



The enantiomers of MFQ can be directly resolved on a (S)-naphthylurea chiral stationary phase using a hexane: 2-propanol: methanol mobile phase with a stereoselectivity (a) factor of 1.63. Due to interferences from endogenous compounds in plasma and whole blood samples, this separation could not be directly applied. To overcome this problem, a coupled achiral - chiral HPLC system was developed (shown in Figure 2) which minimized the analysis time while requiring only one sample injection.

Figure 2: The Achiral-Chiral Coupled Column HPLC System Minimizes Analysis Time.



Waters 600E Multisolvent Delivery System delivers the mobile phase for the cyano column. The high backpressure capabilities of the Waters 484 Tunable Absorbance Detector and minimal bandspreading within the detector cell allow in-line coupling to a second system providing simultaneous assay and chirality measurements.

Apparatus.

The analytical method involved two chromatographic systems connected through a Rheodyne Model 7010 switching valve equipped with a 10 µm silica gel column. The achiral separation was performed with a Waters 600E Multisolvent Delivery System, a manual injector, and a Waters 484 Tunable Absorbance Detector set at 285 nm. The 484 tunable UV detector cell could withstand the back pressure from the second chiral column and the band broadening was minimal allowing quantitation of the total MFQ and the enantiomers with a single injection. The MFQ was separated from interferences in the biological matrix and quantitated on an achiral cyano bonded phase column using the mobile phase specified in Figure 3. By placing the 484 Tunable Absorbance Detector after the achiral column it was possible to detect when the MFQ enantiomers eluted from the achiral column. When this peak eluted from the achiral column it was concentrated on the small guard column and then backflushed to a second isocratic HPLC system using the switching valve. The second HPLC system consisted of an isocratic pump and a variable UV detector set at 285 nm. The stereochemical separation of (+) and (−) MFQ chiral separation was accomplished on a 5 µm (S)-naphthylurea stationary phase. Data from both coupled systems was collected on integrators.

Sample Preparation.

Collection of samples: Blood samples were collected from a healthy male Caucasian volunteer after administration of 15 mg/kg of MFQ base. Blood samples were collected before administration and at various intervals through 30 days. The samples were

separated after collection with 2 ml of whole blood transferred to one polypropylene tube while the remaining sample was centrifuged and the plasma collected and transferred to a separate polypropylene tube. All samples were stored at -20°C until analyzed.

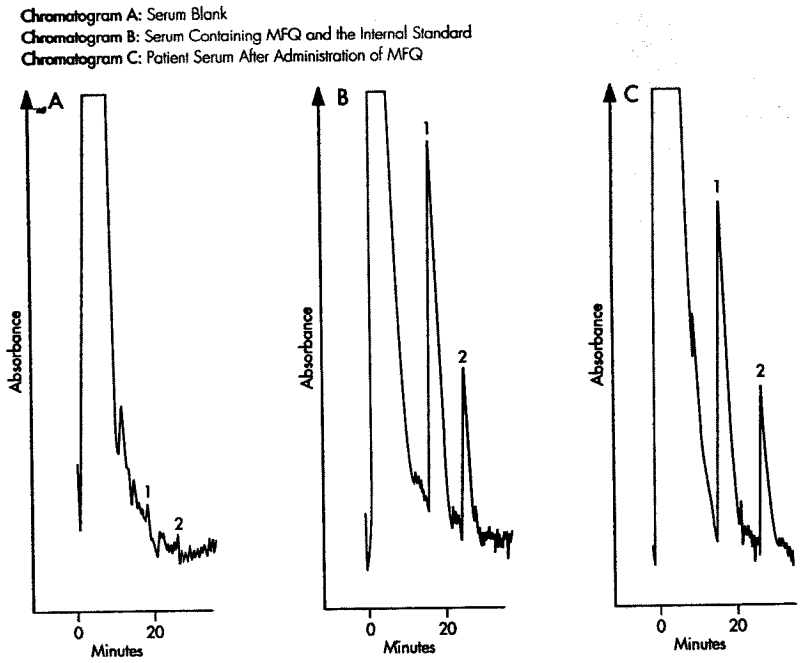
Extraction Procedure and Response Curves.

MFQ was extracted from whole blood and plasma according to Franssen, et. al.,[4]. The sample was concentrated by extraction and evaporation. Prior to injection the sample was dissolved in the mobile phase and analyzed. The recovery and reproducibility of this method was investigated using five drug free serum samples spiked between 0.1 µg/ml and 1.6 µg/ml racemic MFQ with chloroquine (CQ) added as the internal standard to each sample. A standard curve for total MFQ [i.e., (+) and (–) MFQ] plasma concentrations was prepared by the addition of 0.1 to 1.6 µg/ml to drug free serum. The total (+) and (–) MFQ study was carried out on the Waters 600E based chromatographic system with the achiral column and a standard curve was constructed by plotting the MFQ/CQ peak height ratios versus the known MFQ concentrations.

Results and Discussion.

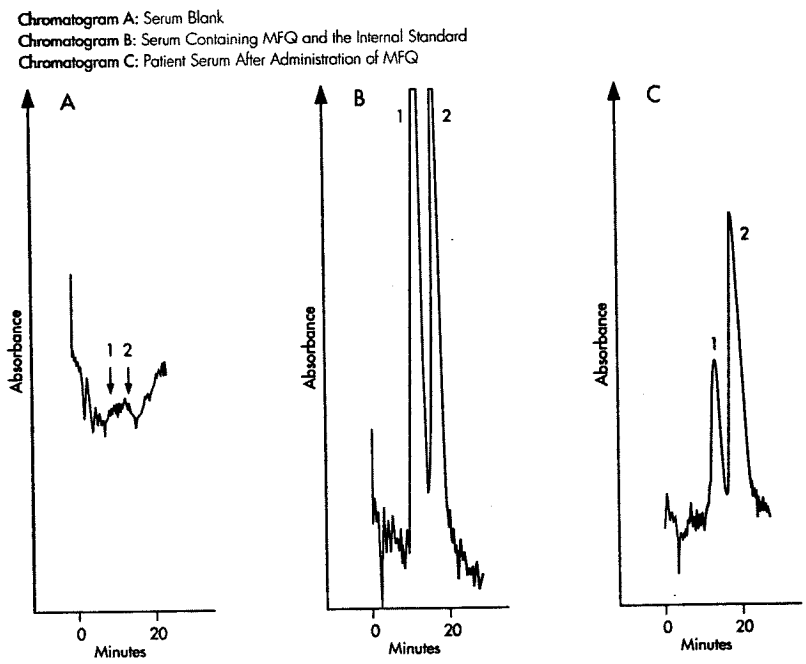
Under the achiral chromatographic conditions used in this study, the chromatographic retention times of MFQ and CQ were 7.6 and 12 minutes, respectively. The MFQ and CQ were well resolved from each other and from interfering compounds from the serum matrix (Figures 3 and 4).

Figure 3: MFQ Separation on an Achiral Cyano Bonded Phase Column.



The cyano column with the mobile phase hexane: 2-propanol: methanol (82:4:14) modified with 0.005% triethylamine separates any biological interferences from the internal standard (peak 2) and the (+,–) MFQ enantiomers (peak 1).

Figure 4: The MFQ Enantiomer Separation on the Chiral Stationary Phase After Initial Separation on the Achiral Cyano Column.



The enantiomer ratio is calculated after separation on the chiral stationary phase using a normal phase mobile phase. The (+) MFQ enantiomer (peak 1) elutes first, followed by the (–) MFQ enantiomer (peak 2).

The standard curve for the MFQ was linear over the range investigated with a correlation coefficient of 0.9993. The limit of detection was 0.05 µg/ml and the percent recovery ranged from 85% at the low concentrations (0.1 µg/ml) to 90% at the high concentrations (1.6 µg/ml).

Representative chromatograms of the MFQ enantiomer separation on the chiral stationary phase are presented in Figure 4. The k's for (+) MFQ and (–) MFQ were 7.9 and 12, respectively. Since baseline resolution of the MFQ enantiomers was not obtained under the chromatographic conditions used in this study, the (+) and (–) MFQ peak height ratio was investigated over the expected plasma concentration range. The observed ratio of 1.1 was used as a correction factor in the calculation of the actual percentage composition of each enantiomer.

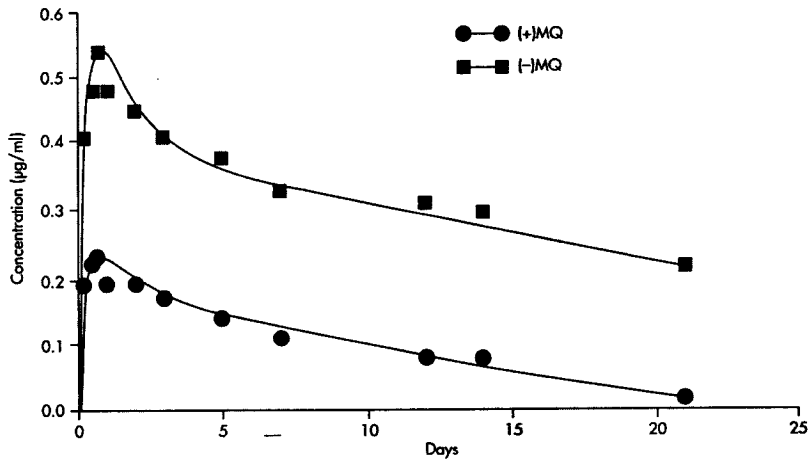
Pharmacokinetic Pilot Study.

The total MFQ concentration was determined on the achiral section of the coupled chromatographic system and the percentages of (+) MFQ and (–) MFQ were determined on the chiral section. Both determinations were carried out in a single experiment with one sample injection minimizing operator time, calculations and correlations.

The plasma concentration versus time curves for the enantiomers of MFQ are presented in Figure 5. The initial results indicate that both the absorption and elimination of the drug are stereospecific. At each experimental point, the plasma concentration of (–) MFQ enantiomer was greater than that of the (+) MFQ enantiomer. The MFQ plasma concentration ratio varied from 1.7 at two hours to 11.5 at 504 hours. Without the stereoselective coupled column chromatography an achiral assay would provide incorrect data on the disposition of this drug.

The whole blood concentration versus time curves for the enantiomers of MFQ also differed, but the magnitude of the difference between the two enantiomers was not as great. The (+) / (–) MFQ whole blood concentration ratio varied from 1.5 at 2 hours to 3 at 504 hours. Results of the complete pnarmacokinetic study are not reported in this article.

Figure 5: Plasma Concentration Versus Time Curves for the Enantiomers of MFQ.



The MFQ was administered as a racemic mixture but the plasma levels differ significantly. To ensure accurate blood level measurements of the active enantiomer, the individual enantiomers must be determined.

Summary.

Chirality has become a major issue in recent years with much work ongoing to determine the enantiomeric ratio and purity of new drugs that contain chiral centers. In addition this work indicates that the pharmacokinetics and stereoselectivity of drug metabolism is of prime importance and cannot be neglected. As shown here, a method that couples achiral and chiral columns can provide added selectivity, specificity and sensitivity, allowing more accurate chiral analysis from challenging sample matrices.

Acknowledgments.

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For more information on Waters 600E Multisolvent Delivery System check box 4 on the attached reply card. For more information on Waters new 486 Tunable Absorbance Detector (replaces the 484 Detector) for coupled achiral-chiral analysis please check box 5 on the attached reply card.