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Note

High-performance liquid chromatographic assay for hydroxychloroquine and three of its major metabolites, desethylhydroxychloroquine, desethylchloroquine and bidesethylchloroquine, in human plasma

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In the late 1940s, hydroxychloroquine sulfate, Plaquenil®★, a 4-aminoquinoline compound, was synthesized and found to be less toxic than, but equally as effective as chloroquine in the treatment of malaria [1—3]. Recently, however, hydroxychloroquine has been used as a therapeutic agent in the treatment of discoid lupus erythematosus and rheumatoid arthritis [4].

Although a number of different analytical methods for the determination of chloroquine and its metabolites have been proposed [5—9], these methods are not selective or sensitive enough for the quantification of hydroxychloroquine and its major metabolites.

In this report we describe a simple, sensitive and selective method, using a reversed-phase high-performance liquid chromatographic (HPLC) procedure utilizing a novel mobile phase for separating hydroxychloroquine, desethylhydroxychloroquine, desethylchloroquine and bidesethylchloroquine in plasma samples (Fig. 1).

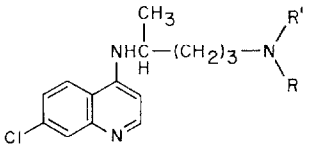

Fig. 1. Chemical structures of hydroxychloroquine (R = CH_2CH_2OH, R' = CH_2CH_3), desethylhydroxychloroquine (R = CH_2CH_2OH, R' = H), desethylchloroquine (R = CH_2CH_3, R' = H), bidesethylchloroquine (R = H, R' = H), chloroquine (R = CH_2CH_3, R' = CH_2CH_3).

★Sterling Drug Inc.'s brand of hydroxychloroquine.

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EXPERIMENTAL

Chemicals and reagents

Hydroxychloroquine sulfate, desethylhydroxychloroquine, desethylchloroquine, bidesethylchloroquine hydrochloride and chloroquine diphosphate (the internal standard) were synthesized at the Sterling-Winthrop Research Institute. All other solvents and chemicals were either of spectroquality or of analytical grade and used without further purification.

Preparation of plasma standards and samples

Plasma standards were prepared by supplementing 1.0 ml of normal human plasma with solutions of each of the above compounds to achieve concentrations of 0 (0.01 *M* hydrochloric acid only), 12.5, 25, 50, 100, 250, 500 and 1000 ng/ml for desethylhydroxychloroquine, desethylchloroquine and bidesethylchloroquine; and 0 (0.01 *M* hydrochloric acid only), 12.5, 25, 50, 100, 250, 500, 1000 and 5000 ng/ml for hydroxychloroquine.

Two sets of randomized and coded samples were prepared for analysis under single-blind conditions, as above. Each plasma set contained triplicate samples at final concentrations of 0, 60, 120, 400 and 800 ng/ml for desethylhydroxychloroquine, desethylchloroquine and bidesethylchloroquine; and 0, 60, 300, 600 and 1200 ng/ml for hydroxychloroquine. One set of plasma samples was analyzed immediately after preparation. The other set was stored in the laboratory freezer for eighteen days prior to analysis.

The observed concentrations for the prepared, spiked samples were expressed as percentage difference from the nominal values. The range of these percentages was used to define the accuracy of the assay. Precision was estimated from the standard deviation derived from the analysis of variance on the percentage differences.

The recoveries (%) of the extraction procedure for the drug, metabolites and the internal standard were determined by comparing the peak heights of hydroxychloroquine, the metabolites and the internal standard obtained from extracted samples with those obtained by direct injection.

Assay method

To a tube containing 1.0 ml of plasma were added 25 μl of the internal standard solution (10 μg/ml in 0.01 *M* hydrochloric acid), 1.0 ml of 1 *M* sodium hydroxide and 6.0 ml of diethyl ether. The samples were agitated on a rotary shaker for 15 min and centrifuged at 825 *g* for 10 min. The organic phase was transferred to a clean silanized 15-ml centrifuge tube. The aqueous phase was again extracted with 6.0 ml of diethyl ether as above. To the pooled organic phase were added 200 μl of 0.01 *M* hydrochloric acid. The tube was stoppered, vortex-mixed and centrifuged at 500 *g* for 10 min. The organic phase was discarded and the aqueous extract was heated at 55—60°C under a stream of nitrogen for 3 min to eliminate residual diethyl ether. An aliquot (100 μl) was injected into the HPLC system for analysis.

Chromatography

The HPLC system was operated isocratically at ambient temperature. The

system consisted of an automatic injector (WISP, Waters Assoc., Milford, MA, U.S.A.), a pump (Model 45, Waters Assoc.), a Zorbax® CN column (15 cm × 4.6 mm I.D., 6 μm particle size, Dupont, Wilmington, DE, U.S.A.) with a 37—50 μm particle size phenyl-Corasil precolumn (Waters Assoc.) and an ultraviolet detector fitted with a 340-nm filter (Model 440, Waters Assoc.). The mobile phase consisted of 0.06 *M* dibutylamine phosphate—0.05 *M* monobasic sodium phosphate, adjusted to pH 3.5 with 85% phosphoric acid (40:60). The flow-rate was 1.2 ml/min. The output of the detector was interfaced with a Model 3357 laboratory automation system (Hewlett-Packard, Palo Alto, CA, U.S.A.) for data acquisition and handling.

Peak-height ratios (parent and metabolites/internal standard) for each standard and sample were calculated and a least-squares regression analysis was performed on the peak-height ratios versus nominal concentration. The concentrations of hydroxychloroquine and its metabolites were determined by inverse prediction from the linear regression [10]. The minimum quantifiable level (MQL) of the assay for all four compounds was defined by the lowest standard (12.5 ng/ml).

RESULTS AND DISCUSSION

The extraction procedure resulted in simple and rapid sample preparation. Representative chromatograms of standards extracted from normal human plasma are shown in Fig. 2A and B. The mean results of the analyses of prepared plasma samples (fresh and frozen) are summarized in Table I. Plots of peak-height ratios (drug/internal standard) versus concentration for the plasma standards were linear over the range 0—5000 ng/ml of plasma for hydroxychloroquine (typically, $y = 0.007x - 0.034$, $r = 0.9999$) and 0—1000 ng/ml of plasma for each of the metabolites ($y = 0.019x + 0.001$, $r = 0.9998$ for desethylhydroxychloroquine; $y = 0.017x + 0.016$, $r = 0.9999$ for desethylchloroquine; $y = 0.033x + 0.004$, $r = 0.9997$ for bidesethylchloroquine).

The assay precision was 4.31% for hydroxychloroquine, 2.79% for desethyl-

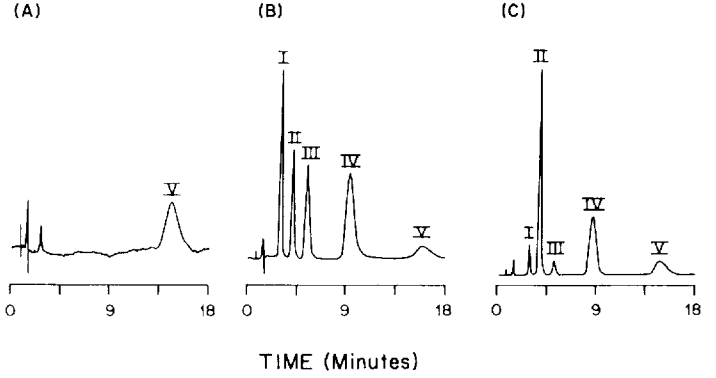

Fig. 2. Representative chromatograms of (A) an extracted plasma blank with internal standard only; (B) an extracted plasma standard containing 500 ng/ml each of bidesethylchloroquine (I), desethylhydroxychloroquine (II), desethylchloroquine (III) and hydroxychloroquine (IV) and 250 ng/ml internal standard (V); and (C) an extracted plasma from patient 1 (Table III) following daily oral administration of 400 mg of hydroxychloroquine.

TABLE I

RESULTS OF THE ANALYSIS OF PREPARED PLASMA SAMPLES (n = 6)

Nominal concentration (ng/ml)	Mean observed concentration (ng/ml)	Mean percentage difference	Standard error of the mean	Coefficient of variation (%)
Hydroxychloroquine				
60	61.5	2.5	1.0	4.0
300	291	−3.1	5.9	4.9
600	610	1.7	11.1	4.5
1200	1190	−0.61	8.1	1.7
Desethylhydroxychloroquine				
60	60.3	0.42	1.0	4.0
120	121	0.42	0.89	1.8
400	405	1.2	4.9	3.0
800	798	−0.31	7.5	2.3
Desethylchloroquine				
60	59.8	−0.28	1.1	4.4
120	123	2.1	1.8	3.5
400	402	0.54	4.6	2.8
800	818	2.3	10.6	3.2
Bidesethylchloroquine				
55	54.7	−0.52	0.67	3.0
120	120	0.00	2.4	4.8
400	401	0.25	8.1	4.9
800	795	−0.69	13.5	4.2

TABLE II

RESULTS FROM LINEAR REGRESSION ANALYSIS OF OBSERVED VERSUS NOMINAL CONCENTRATIONS FOR FRESH AND FROZEN SAMPLES

Compound	Slope	Intercept (ng/ml)	Coefficient of determination (R^2)
Hydroxychloroquine			
Fresh	0.999	−1.65	0.998
Frozen	0.994	3.32	0.998
Desethylhydroxychloroquine			
Fresh	0.982	4.91	0.999
Frozen	1.01	−1.17	0.999
Desethylchloroquine			
Fresh	1.04	−7.53	0.997
Frozen	1.00	3.09	0.999
Bidesethylchloroquine			
Fresh	0.962	2.24	0.999
Frozen	1.02	0.093	0.998

plaquenil, 3.51% for desethylchloroquine and 4.03% for bidesethylchloroquine. The accuracy of the assay varied from —3.1% to 4.9% for hydroxychloroquine, —2.9 to 3.7% for desethylhydroxychloroquine, —2.6 to 3.9% for desethylchloroquine and —3.6 to 4.1% for bidesethylchloroquine.

Regression analyses of the observed versus nominal concentrations of the triplicate fresh and frozen samples revealed no appreciable difference in the slopes, intercepts or coefficients of determination (Table II).

The extraction efficiency was 85% for hydroxychloroquine, 92% for desethylhydroxychloroquine, 90% for desethylchloroquine, 86% for bidesethylchloroquine and 88% for the internal standard (chloroquine).

Plasma and serum samples from patients receiving daily oral doses of either 200 or 400 mg of hydroxychloroquine were analyzed by the above method. Blood samples were taken by venipuncture. The plasma or serum was separated by centrifugation and stored in the laboratory freezer until analyzed.

The results of the analysis are given in Table III. The times of the last drug administration and sample collection times are not available so these samples serve only to demonstrate the utility of the method for quantifying hydroxychloroquine and its major metabolites. Fig. 2C is a chromatogram of an extracted patient plasma following daily oral administration of 400 mg of hydroxychloroquine.

TABLE III

PLASMA CONCENTRATIONS OF HYDROXYCHLOROQUINE AND THREE OF ITS METABOLITES AFTER CHRONIC ORAL ADMINISTRATION OF HYDROXYCHLOROQUINE

Patient	Concentration (ng/ml)			
	Hydroxychloroquine	Desethylchloroquine	Desethylhydroxychloroquine	Bidesethylchloroquine
1	625	71.3	837	72.5
2	290	47.8	229	20.4
3	87.1	<MQL*	21.7	<MQL
4	66.6	24.8	34.4	<MQL
5	220	30.4	155	16.1
6	210	22.6	166	<MQL

*Minimum quantifiable level (MQL) = 12.5 ng/ml.

CONCLUSION

In summary, an accurate, selective, reproducible and precise HPLC assay has been developed for the determination of hydroxychloroquine, desethylhydroxychloroquine, desethylchloroquine and bidesethylchloroquine concentrations in human plasma. This method has proven useful for analysis of specimens obtained during clinical use.

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