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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE SIMULTANEOUS MONITORING OF MEFLOQUINE AND ITS ACID METABOLITE IN BIOLOGICAL SAMPLES USING PROTEIN PRECIPITATION AND ION-PAIR EXTRACTION

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SUMMARY

A high-performance liquid chromatographic (HPLC) method is presented for the simultaneous determination of mefloquine and its acid metabolite in plasma and whole blood. Plasma and whole blood are deproteinized with a combination of zinc and acetonitrile before extraction. Mefloquine and its acid metabolite are extracted simultaneously at pH 4 by methyl *tert.*-butyl ether, where mefloquine is extracted as an ion pair with heptanesulphonate. After evaporation of the organic phase, the residue is dissolved in mobile phase and injected on to the chromatographic column. A reversed-phase column (Spherisorb ODS-1) is used with acetonitrile–phosphate buffer (0.1 mol/l, pH 2.5) (42:58) containing 40 mmol/l perchlorate as the mobile phase. N,N-Dioctylamine was added to the mobile phase to give a concentration of 0.1% and the pH was adjusted to 2.3–2.7 with concentrated phosphoric acid. The method permits the determination of 0.10 μmol/l (30 ng/ml) mefloquine and its acid metabolite in plasma. The coefficient of variation was 5–6% at the therapeutic level (mefloquine 1–4 μmol/l, its carboxylic metabolite 2–6 μmol/l) in 0.5-ml samples. An alternative method is also described with a similar clean-up procedure that uses protein precipitation with zinc–acetonitrile as a sample pretreatment for therapeutic monitoring of mefloquine and metabolite in plasma and whole blood. Using this method, 0.25 μmol/l mefloquine and its metabolite can be determined. The results from the two methods correlate well.

INTRODUCTION

Mefloquine [DL-*erythro*-α-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinoline-

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methanol] (MQ) (Fig. 1) is a quinolylmethanol derivative active against all malaria species in humans [1]. The main metabolite of MQ is 2,8-bis(trifluoromethyl)-4-quinolinecarboxylic acid (MMQ) (Fig. 1.). With widespread chloroquine-resistant *Plasmodium falciparum* in most malarious areas and increasing resistance to sulphonamide–pyrimethamine combinations, MQ is one of the few effective drugs available [2,3]. High-performance liquid chromatographic (HPLC) and gas chromatographic (GC) methods for the determination of MQ have been described [4–9]. The GC methods [4–6] for determining MQ in biological fluids use derivatization procedures. The detection limits for the above methods are between 2.6 and 26 nmol/l using sample volumes in the range 0.1–1.0 ml. A practical application of the phosgene derivatization reaction is used in a new GC method [7] and concentrations down to 25 nmol/l can be determined in 100 μl of plasma or whole blood. Its application to the assay of MQ in capillary blood collected on filter-paper was also presented [7]. The current methods for the simultaneous assay of MQ and MMQ are thin-layer chromatography [10] and, recently HPLC involving direct injection of plasma with precolumn enrichment and use of the column switching technique [11]. The coefficient of variation in plasma at the 10 nmol/l level of MQ and MMQ were about 2.4% [11]. Another HPLC procedure for MQ is available but has not yet been published in detail [12]. Owing to the absence of sufficiently sensitive and specific analytical methods for monitoring the levels of MQ and MMQ in biological fluids, little is known about their whole blood concentrations. Whole blood samples are easier to obtain in the field and might therefore be preferable for clinical studies. In a recent study on patients treated with a combination of MQ, sulphadoxine and pyrimethamine using an HPLC method [12] with an extraction procedure with modification for whole blood samples, the plasma levels of MQ during the first 48 h corresponded well with the whole blood concentrations [13]. However, MMQ concentrations were not determined.

In this study, we combined an efficient ion-pair extraction method and an HPLC system [14] in the reversed-phase mode, permitting the simultaneous determination and separation of MQ and MMQ using an analogue of MQ as an internal standard. This method was evaluated by using blood samples from volunteers given MQ as a prophylactic and from monkeys given MQ treatment.

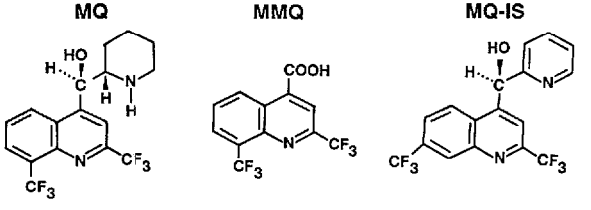

Fig. 1. Structures of mefloquine (MQ), the metabolite of MQ (MMQ) and the internal standard (MQ-IS).

EXPERIMENTAL*

Reagents and chemicals

HPLC-grade acetonitrile and methyl *tert.*-butyl ether (MTBE) were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.), diethyl ether and methylene chloride (MDC) from Merck (Darmstadt, F.R.G.), heptanesulphonic acid from Sigma (St. Louis, MO, U.S.A.) and N,N-dimethyloctylamine (DMOA) from Aldrich (Milwaukee, WI, U.S.A.). MQ, MMQ and *rac.*-α-(2-pyridyl)-2,7-bis(trifluoromethyl)-4-quinoline (MQ-IS) (Fig. 1) were kindly supplied by Roche-Produkter (Skarholmen, Sweden). All other chemicals were of analytical-reagent grade. Stock solutions at a concentration of 500 μmol/l of MQ and MQ-IS were dissolved in 10 mmol/l hydrochloric acid and MMQ in 10 mmol/l sodium hydroxide solution. The appropriate working solutions (200 μmol/l) were freshly prepared each week by dilution of the stock solutions with deionized water. These solutions (MQ, MMQ) were used for plasma or whole blood spiking. To test the accuracy of the dilution and the stability of stock and working standard solutions, the molar absorptivity (ε) was determined at intervals. For MQ $\varepsilon = 5400 \pm 67$ l mol^{-1} cm^{-1} in 4 mmol/l hydrochloric acid and for MMQ $\varepsilon = 5800 \pm 65$ l mol^{-1} cm^{-1} in 4 mmol/l sodium hydroxide solution, both at 290 nm.

Glassware and polypropylene tubes were washed with general laboratory detergent, allowed to stand overnight in 2 mol/l hydrochloric acid to minimize adsorption of the drugs, rinsed with deionized water and then acetone and dried.

Instruments and chromatographic conditions

The chromatographic system consisted of a Model SP8700XR HPLC pump (Spectra-Physics, San Jose, CA, U.S.A.) and WISP 710B autoinjector (Waters Assoc., Milford, MA, U.S.A.) with a Spectroflow 757 (Kratos, Ramsey, NJ, U.S.A.) variable-wavelength UV detector set at 222 nm. A 175 mm × 4.6 mm I.D. stainless-steel column was laboratory-packed with 5-μm Spherisorb ODS-1 (Phase Separations, Queensferry, U.K.) The peaks were evaluated with a Model SP4270 computing integrator (Spectra-Physics). The flow-rate through the column, at ambient temperature, was 1–1.5 ml/min. The mobile phase was acetonitrile–phosphate buffer (0.1 mol/l, pH 2.5) (42:58) containing 40 mmol/l perchlorate. N,N-Dimethyloctylamine was added to the mixture to give a 0.1% concentration and the pH was adjusted with phosphoric acid to achieve baseline separation between the compounds; using Spherisorb ODS-1 as the support, this pH value was between 2.30 and 2.70. A microcentrifuge (Haake Buckler Instruments, Saddle Brook, NJ, U.S.A.) was used to centrifuge samples after protein precipitation.

Ion-pair extraction method

Plasma, whole blood and spiked plasma or whole blood standards (0.3–0.5 ml) containing both MQ (0.1–5.0 μmol/l) and MMQ (0.5–10 μmol/l) were vortex-mixed for 10 s in polypropylene tubes during which 100 μl of 0.25 *M* zinc sulphate

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solution were added dropwise. A 1-ml volume of acetonitrile containing MQ-IS (5 μmol/l) as internal standard was added in the same manner during vortex-mixing. Mixing was continued for 30 s. After standing for 15 min the tubes were centrifuged at 10 000 *g* for 7 min. The supernatant was decanted into polypropylene tubes and 3 ml of 0.1 *M* heptanesulphonic acid in 0.1 mol/l acetate buffer (pH 4.0) and 6 ml of MTBE were added. The mixture was shaken for 30 min and then centrifuged at 1250 *g* for 5 min. The organic layer was transferred into a conical polypropylene tube and evaporated to dryness at 50 °C under a stream of nitrogen. The residue was reconstituted in 300 μl of mobile phase via ultrasonication (3 min) and 100 μl were injected into the HPLC system. A simultaneous extraction of MQ and MMQ can also be accomplished at pH 9.00 with a quaternary amine, such as the tetrahexylammonium ion, to form an ion pair with MMQ [14]. However, the quaternary amine gives a large solvent peak at both 222 and 285 nm in the chromatographic system.

Protein precipitation method (without ion-pair extraction)

A 200-μl volume of plasma, whole blood or spiked standards, 50 μl of MQ-IS (30 μmol/l) and 50 μl of 0.2 *M* zinc sulphate solution were added dropwise to polypropylene tubes during vortex-mixing for 30 s. A 500-μl volume of acetonitrile was then added dropwise during vortex-mixing for 30 s. After 15 min the tubes were centrifuged at 10 000 *g* for 7 min. The supernatant was decanted into polypropylene tubes and evaporated to dryness at 55–60 °C for 15–20 min under a stream of nitrogen. The residue was reconstituted in 200 μl of mobile phase via ultrasonication (3 min) and 125 μl were injected into the HPLC system. If the solution became turbid, further centrifugation at 10 000 *g* for 5–7 min was necessary.

Before the assay, whole blood was haemolysed by dilution with water (1:1) followed by freezing in both the ion-pair extraction and protein precipitation methods.

Recovery and assay precision

The absolute recovery of simultaneously extracted MQ and MMQ was calculated by comparing the peak heights of injected spiked plasma and whole blood extracts with those obtained from injecting pure solutions of MQ and MMQ prepared in mobile phase. The intra- and inter-assay precision were determined by analysis of spiked plasma and whole blood at different concentrations.

Heat treatment of spiked MQ and MMQ samples

Plasma and whole blood were spiked with MQ and MMQ at therapeutic concentrations. The samples were divided into six portions, three being heated in a water-bath at 56 °C for 30 min in screw-capped glass bottles and the other remaining three not being heated. The heated samples were allowed to cool and the concentrations of MQ and MMQ were assayed by the ion-pair extraction method in the same batch as the corresponding untreated samples.

Application of the assay

To demonstrate the applicability of the ion-pair extraction method, whole blood samples from monkeys (*Saimiri sciureus*) were analysed. Single doses of 25–30

mg/kg body weight MQ were administered orally. Serial blood samples were taken up to five weeks after dosing. Whole blood and plasma samples were obtained from a clinical study in which subjects were undergoing MQ prophylaxis (250 mg per week). These samples were used to determine the correlation of the extraction and protein precipitation methods.

RESULTS AND DISCUSSION

Deproteinization

To determine the whole blood concentration of MQ at low pH with the ion-pair extraction technique, a deproteinization step is necessary in order to eliminate haemoglobin-derived material, which gives a brown colour in the organic phase after extraction. There are problems with endogenous peaks when applying an ion-pair extraction technique to plasma and whole blood, particularly when a wavelength of 222 nm is used for detection. These interactions can be avoided by removal of the plasma proteins from the samples before the extraction, thus achieving higher sensitivity. The use of perchloric acid and trichloroacetic acid for deproteinization resulted in a low recovery of MMQ. Precipitation techniques with heavy metals were also unsuccessful. The best results were obtained with a combination of zinc and acetonitrile. Zinc sulphate was added dropwise during continuous vortex-mixing. Acetonitrile was added in the same manner. Incomplete protein precipitation resulted, however, when either zinc or acetonitrile was used alone as the protein precipitant. Zinc forms "complexes" with proteins [15], and these complexes dehydrate on addition of acetonitrile and precipitate from solution. In the extraction method, protein precipitation with zinc decreases the recovery of both MQ and MMQ (Fig. 2). This decrease was not seen when using the protein precipitation method (Table I). The decrease is more pronounced for MMQ and for MQ. To obtain a "clean" protein precipitate, the optimum concentration of zinc sulphate added during the deproteinization step is between 5 and 10 mmol/l for plasma and between 10 and 20 mmol/l for whole blood. The chromatographic and extraction conditions that produce a good separation for MQ, MMQ and MQ-IS, with reasonable retention times, were investigated [14]. MTBE was used as the extraction solvent because the phases separated more easily with this solvent than with methylene chloride. MTBE is resistant to peroxide formation and a good substitute for diethyl ether. Interference from endogenous plasma and whole blood constituents was minimized by protein precipitation before the extraction.

Linearity and precision

Calibration graphs obtained using six different concentrations between 0.25 and 8 μmol/l prepared in drug-free plasma or whole blood were linear. A representative example of a plasma curve for MQ and MMQ is presented in Table II. The protein precipitation method had a lower sensitivity (> 0.25 μmol/l). A high-speed centrifuge and sensitive detection at 222 nm are also necessary. A statistical comparison of the methods was made for human plasma samples, where y represents the protein precipitation results and x the ion-pair extraction results: for

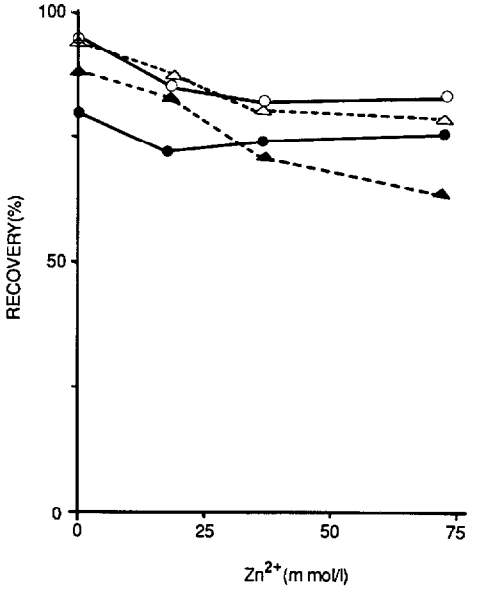

Fig. 2. Effect of zinc sulphate concentration on the recovery in the deproteinization step, before ion-pair extraction. Analytes: (○) MQ in plasma; (●) MQ in whole blood; (△) MMQ in plasma; (▲) MMQ in whole blood.

TABLE I

RECOVERY OF MQ AND MMQ FROM PLASMA AND WHOLE BLOOD

Sample	Concentration (mmol/l)	Recovery (%)	
		MQ	MMQ
Extraction method			
Plasma	1.25	88.6±5.5 (n=7)	–
	5.00	–	78.7±7.0 (n=6)
Whole blood	1.25	73.8±11.5 (n=5)	–
	5.00	–	69.0±5.5 (n=6)
Protein precipitation method			
Plasma	0.50	88.0±4.3 (n=5)	89.7±11.5 (n=5)
	1.00	91.2±8.6 (n=3)	–
	3.00	90.4±4.1 (n=6)	88.2±9.5 (n=3)
	7.00	–	90.3±3.9 (n=9)
Whole blood	1.00	87.6±6.5 (n=5)	80.8±5.0 (n=5)
	3.00	89.6±5.9 (n=9)	–
	7.00	–	95.4±6.5 (n=9)

MQ $y=1.01x+0.095$ ($n=10$), $r=0.988$; for MMQ $y=0.94x+0.021$ ($n=10$), $r=0.980$. Other analytical evaluations of the two methods are given in Tables I and III.

TABLE II

LINEARITY OF A REPRESENTATIVE CALIBRATION GRAPH FOR PLASMA (EXTRACTION METHOD)

Spiked calculated concentration (μmol/l)	MQ		MMQ	
	Peak-height ratio	Calculated concentration (μmol/l)	Peak-height ratio	Calculated concentration (μmol/l)
0.25	0.33	0.34	0.15	0.27
0.5	0.79	0.59	0.29	0.48
1	1.36	0.91	0.60	0.94
3	4.97	2.91	2.00	3.04
5	8.55	4.89	3.35	5.06
8	14.35	8.10	5.28	7.95
Correlation, r	0.999		0.999	
Slope, b	1.81		0.668	
Intercept, a	−0.282		0.0302	

Recovery and precision

The recoveries of MQ and MMQ by the extraction method were 80–90% in plasma and 70–75% in whole blood (Table I). Some loss of drug is expected because of difficulties in removing all the solvent in the aqueous liquid interface and in the protein precipitation step. The intra- and inter-assay precisions are given in Table III.

Selectivity and accuracy

Fig. 3 shows typical chromatograms obtained from MQ and MMQ dissolved in mobile phase, drug-free plasma spiked with MQ, MMQ and MQ-IS and patient plasma with and without MQ-IS. The interfering substances in biological fluids are eluted in the solvent front. With the present chromatographic system, no interference from other commonly used antimalarial drugs or metabolites was seen [14]. The present methods can therefore be used when patients are treated with other antimalarial drugs in combination with MQ. A comparison of the present method and the GC method [7] for the assay of MQ in plasma and whole blood samples gave the regression equation $y=1.015x-0.32$ with a correlation $r=0.977$ ($n=17$). Samples were taken from subjects on mefloquine prophylaxis (250 mg per week).

Limit of determination

The limit of determination of the methods depends on the initial sample volume used. When using the extraction method, the limit of determination is 0.10 μmol/l for plasma and 0.5 μmol/l for whole blood for both MQ and MMQ at 220 nm using a 300-μl sample volume. For the protein precipitation method, the initial sample volume should be 200 μl, otherwise endogenous peaks are sufficiently large to interfere with the chromatographic separation. The limit of determination with protein precipitation is 0.25 μmol/l for both plasma and whole blood

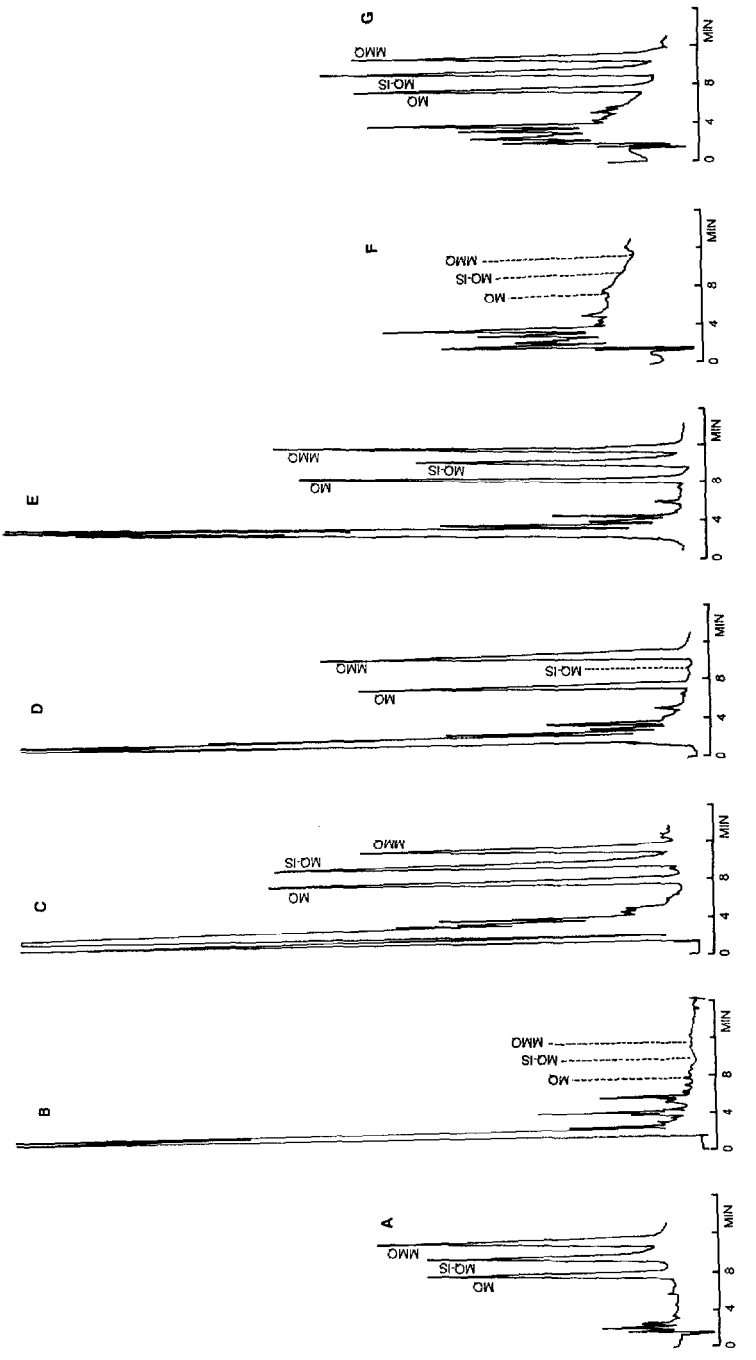

Fig. 3. Chromatograms obtained. Extraction method: (A) drugs dissolved in mobile phase, 5.0 μmol/l of each drug; (B) drug-free human plasma sample (retention times for the drugs are indicated by the dotted lines); (C) spiked human plasma sample with MQ (2.0 μmol/l), MMQ (3.0 μmol/l) and internal standard (MQ-IS); (D) patient plasma sample without MQ-IS (retention time for MQ-IS at the dotted line); (E) patient plasma sample with MQ-IS, MQ = 2.8 μmol/l and MMQ = 5.9 μmol/l. Protein precipitation method: (F) drug-free human plasma sample (retention time for the drugs are indicated by the dotted lines); (G) patient plasma sample, MQ = 2.8 μmol/l and MMQ = 5.9 μmol/l. Mobile phase, acetonitrile–phosphate buffer (0.1 mol/l, pH 2.50) (42:58) containing 40 mmol/l perchlorate (0.1% DMOA, adjusted to pH of 2.3): flow-rate, 1.3 ml/min; stationary phase, Spherisorb ODS-1.

TABLE III

INTRA- AND INTER-ASSAY PRECISION OF MQ AND MMQ IN PLASMA AND WHOLE BLOOD

Sample	Concentration (μmol/l)	Coefficient of variation (%)	
		MQ	MMQ
Extraction method, intra-assay			
Plasma	0.10	11.0 ($n=5$)	17.5 ($n=4$)
	0.50	2.5 ($n=4$)	–
	1.25	4.5 ($n=5$)	–
	3.48	–	3.7 ($n=8$)
	6.34	–	4.9 ($n=6$)
Whole blood	0.50	9.3 ($n=5$)	12.4 ($n=5$)
	1.59	5.7 ($n=9$)	–
	3.31	–	6.0 ($n=9$)
Extraction method, inter-assay			
Plasma	0.97	12.7 ($n=9$)	–
	4.93	–	8.7 ($n=13$)
Protein precipitation method, intra-assay			
Plasma	0.25	11.4 ($n=5$)	10.4 ($n=5$)
	1.00	4.1 ($n=5$)	3.8 ($n=5$)
	3.00	4.6 ($n=6$)	–
	7.00	–	4.3 ($n=6$)
Whole blood	0.25	13.7 ($n=5$)	11.1 ($n=5$)
	1.00	6.5 ($n=5$)	3.0 ($n=5$)
	3.00	5.2 ($n=10$)	–
	5.00	–	3.7 ($n=5$)
	7.00	–	7.4 ($n=10$)

samples. Below these concentrations the intra-assay standard deviation is greater than 18%.

Effect of heat treatment

Laboratory personnel are concerned about the risks of exposure to human immunodeficiency virus (HIV). HIV is thermolabile and is inactivated by heating at 56°C for 30 min [16,17]. The effect of heating spiked plasma and whole blood of MQ and MMQ is shown in Table IV. The concentrations of MQ and MMQ were not altered, within the precision of the assay, on heating at 56°C for 30 min.

Application

MQ whole blood levels in 500-μl samples using the extraction method can be monitored in monkeys up to five weeks after a single oral dose of 25–30 mg/kg body weight MQ (Fig. 4). The metabolite (MMQ) was not produced in these monkeys to the same extent as in humans. However, a component with a retention time less than MQ appeared. This component was absent in the pre-dose sample and may be an unidentified metabolite of MQ in monkey samples that is not found in human samples using this method.

TABLE IV

EFFECT OF HEAT TREATMENT AT 56°C

Sample	Compound	Concentration (mean ± S.D., $n=3$) (μmol/l)	
		0 min	30 min
Plasma	MQ	1.63 ± 0.10	1.62 ± 0.12
	MMQ	3.44 ± 0.16	3.46 ± 0.11
Whole blood	MQ	1.50 ± 0.02	1.56 ± 0.04
	MMQ	3.80 ± 0.12	3.91 ± 0.04

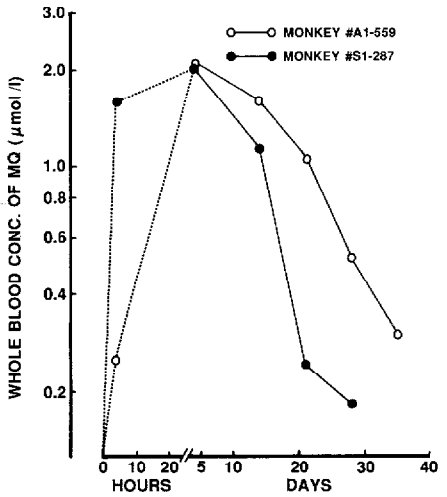

Fig. 4. Time course of MQ levels in blood samples from two monkeys after oral administration of 25–30 mg/kg of MQ.

CONCLUSION

Two alternative methods for the simultaneous determination of MQ and its carboxylic metabolite, MMQ were investigated. A simple protein precipitation method was evaluated. Using this method, high-speed centrifugation is necessary to eliminate the protein precipitation agents which would otherwise interfere when a detection wavelength of 222 nm is used. This wavelength is necessary to achieve sufficient sensitivity for therapeutic monitoring. The extraction method has a higher sensitivity (0.10 μmol/l) for both MQ and MMQ in plasma samples. The methods correlate well with each other at the therapeutic levels for MQ and MMQ. To determine the low concentrations (<0.25 μmol/l) of MQ for pharmacokinetic studies and finger-stick blood samples collected on filter-paper and also urine samples, we suggest a recently developed GC method [17].

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