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Stereospecific determination of mefloquine in biological fluids by high-performance liquid chromatography

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Abstract

A sensitive stereoselective HPLC method was developed for determination of mefloquine (MFQ) enantiomers in plasma, urine and whole blood. The assay involved liquid–liquid extraction of MFQ from biological fluids with a mixture of hexane and isopropanol in the presence of sodium hydroxide and derivatization of the residue by (+)-(*S*)-naphthylethylisocyanate (NEIC) as chiral derivatizing reagent. Separation of the resulting diastereomers was performed on a silica normal-phase column using chloroform–hexane–methanol (25:74:1) as the mobile phase with a flow-rate of 1 ml/min. Using 200 μl of plasma or whole blood, the limit of determination was 0.2 μg/ml with UV detection for both enantiomers. The limit of determination in 500 μl of urine was 0.08 μg/ml with UV detection. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Mefloquine

1. Introduction

Mefloquine (MFQ), *rac-erythro*-α-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol (Fig. 1), is a chiral drug administered orally as a racemate mixture for prophylaxis and treatment of malaria caused by multiple-drug-resistant strains of *Plasmodium falciparum*. There are conflicting reports about the antimalarial activity of MFQ enantiomers. In some reports, no significant difference is observed between antimalarial activities of enantiomers against *Plasmodium berghei* in rodents [1] and against human *Plasmodium falciparum* in vitro [2]. According to Karle et al. [3], the (+)-(*RS*)-enantiomer of MFQ was 1.69–1.81 times more active than the (−)-(*SR*)-enantiomer against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* in vitro. Ngiam and Go [4] found that the (−)-(*SR*)-enantiomer is a more potent inhibitor of acetylcholinesterase and butyrylcholinesterase than the (+)-(*RS*)-enantiomer. Pharmacokinetic studies of MFQ enantiomers have also shown high stereoselectivity in humans [5–7].

Qiu et al. [8] reported a stereoselective HPLC method using a Chiralpak AD column for determination of all four stereoisomers of *erythro* and *threo* forms of MFQ in commercial tablets without any validation data in biological fluids. Gimenez and co-workers [5,9] reported a column-switching HPLC method for the analysis of MFQ enantiomers using a double achiral–chiral system of chromatography. This method is complicated and has a determination limit of 0.05 μg/ml in 500 μl of plasma. Wallen et al. [10] reported an enantioselective method using a

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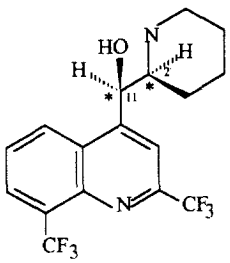
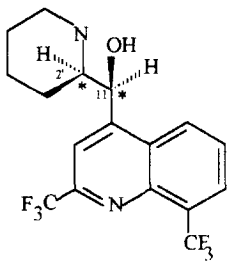
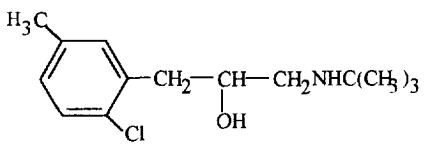


(+)-Mefloquine
11R, 2'S



(-)-Mefloquine
11S, 2'R



rac-Bupranolol

Fig. 1. Chemical structure of mefloquine (MFQ) and internal standard.

chiral AGP column. Bergqvist et al. [11] used a chiral counter ion in mobile phase to determine MFQ enantiomers in plasma. The limit of determination of the latter two methods were 0.1 and 0.5 μmol/l in either 1000 or 300 μl of samples. An indirect method using (−)-1-(9-fluorenyl)ethyl chloroformate as derivatizing reagent with a 40-min reaction time was performed by Bergqvist et al. [12] for analysis of MFQ enantiomers in biological fluids with both UV and fluorescence detectors.

The aim of this study was to develop a stereoselective HPLC method for the determination of (−)-(*SR*)- and (+)-(*RS*)-MFQ enantiomers in biological fluids using an inexpensive derivatization reagent and a non-chiral column, and could be used in stereoselective pharmacokinetic studies in human and animal models.

2. Experimental

2.1. Chemicals and reagents

Racemic MFQ-HCl was purchased from Roche (Basel, Switzerland). The (−)-(*SR*)- and (+)-(*RS*)-MFQ enantiomers were resolved with (+)-3-bromo-8-camphorsulphonic acid ammonium salt (Aldrich, Milwaukee, WI, USA) according to Carrol and Blackwell [1]. Internal standard, *rac*-bupranolol (I.S.) (Fig. 1), was from Logeais (Issy-les-Molineaux, France). The derivatizing reagent, (+)-(*S*)-naphthylethylisocyanate (NEIC) was purchased from Aldrich (Milwaukee, WI, USA). The optical purity of the reagent was higher than 99.5%. Hexane, chloroform, methanol and isopropanol were HPLC grade products from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals and solvents were of analytical reagent grade and used without any further purification.

2.2. Standard solutions

Stock standard solution of *rac*-MFQ was prepared by dissolving MFQ-HCl in methanol to a final concentration of 40 μg/ml (solution 1). Standard solutions 2–6 containing 1.6, 3.2, 4, 8 and 12 μg/ml were obtained by subsequent dilution of aliquots of solution 1 with methanol.

A solution of I.S. was prepared by dissolving bupranolol hydrochloride in 0.01% triethylamine in chloroform to a final concentration of 20 μg/ml. All these solutions were stored at 4°C.

A solution of 0.1% (v/v) (+)-(*S*)-NEIC was prepared in chloroform and stored at −20°C until just prior to use.

2.3. Instrumentation and chromatography

Samples were vortexed using a Vortex Geniez mixer (Fisher Scientific, Edmonton, Alb., Canada). Evaporation of samples were done on a Savant Vac cocentrator-evaporator (Emerston Instruments, Scarborough, Ont., Canada).

The chromatographic system consisted of a 590 HPLC pump, a WISP 710B autoinjector and a variable 481 UV detector all from Waters (Mississauga, Canada). The recorder-intergrator was a Hewlett-Packard (Palo Alto, CA, USA).

The reversed-phase system was a modified method of Bergqvist et al. [13] for determination of *rac*-MFQ and consisted of a Partisil 5 ODS column (250 mm×4.6 mm, Phenomenex, Torrance, CA, USA) and acetonitrile–phosphate buffer, pH 3.5 (51:49,

v/v) was used as eluent and pumped at a flow-rate of 1.4 ml/min. Detection was performed at 229 nm.

The normal-phase system consisted of a Partisil 5 (Silica) column (250 mm×4.6 mm, Phenomenex, Torrance, CA, USA) and a mobile phase of hexane–chloroform–methanol (74:25:1, v/v/v) pumping at a flow-rate of 1 ml/min. Detection was performed at 282 nm.

2.4. Extraction procedure

The plasma sample (200 μl) and I.S. solution (50 μl) were added to a 10-ml disposable siliconized glass test tube, and vortex-mixed for 15 s. Then, 500 μl of 0.2 *M* sodium hydroxide and 3 ml of hexane–isopropanol (95:5, v/v) were added, the test tube was vortex-mixed for 1 min and centrifuged at 700 *g* for 15 min. The organic layer was transferred by Pasteur pipet to a clean siliconized 10-ml test tube and evaporated to dryness.

Blood samples were subjected to three cycles of freeze–thaw lysis using a mixture of dry ice in acetone after adding the sodium hydroxide solution and left at room temperature for 10 min before extraction by organic solvent.

Extraction of 500-μl urine samples were carried out as described for plasma.

2.5. Derivatization

The extraction residue was reconstituted in 300 μl of chloroform and 20 μl of 0.1% (+)-(*S*)-NEIC in chloroform was added. After mixing, the sample was capped and left at ambient temperature for 20 min. The mixture was evaporated to dryness. The residue was redissolved in 300 μl of the mobile phase and 50 μl were injected into the HPLC system.

2.6. Derivatization yield

Mefloquine (solution 6) was added to clean test tubes ($n=12$) to give a concentration of 1.5 μg/ml in 0.01% (v/v) triethylamine in chloroform. Six samples were derivatized as described above. The amount of underivatized MFQ was calculated by comparing the peak areas against six underivatized samples using a reversed-phase HPLC method described previously. The experiment was repeated on three consecutive days.

2.7. Extraction yield

Aliquots of 50 μl of standard solutions of MFQ (solutions 2 and 6) were added separately to two sets of six test tubes. Then I.S. solutions (50 μl) were added and evaporated to dryness. The residues were reconstituted in 0.2 ml of plasma to give concentrations of 0.2 and 1.5 μg/ml of each enantiomer and left at room temperature for 20 min to reach to equilibrium. After extraction, exactly 2.5 ml of the organic layers of each tube were placed in clean tubes, evaporated and derivatized. The peak areas of extracted MFQ and I.S. diastereomers were compared by unextracted equivalent concentrations of MFQ and I.S. under identical chromatographic conditions. The experiment was repeated on three consecutive days. The same experiments were done for whole blood and urine.

2.8. Sample collection

To test the applicability of the analytical method to pharmacokinetic studies, the plasma and whole blood levels of MFQ enantiomers were measured in rats after administration of *rac*-MFQ. A rat underwent surgical cannulation of the right jugular vein under anesthesia with methoxyflurane and allowed to recover overnight. A 50 mg/kg oral dose of racemic MFQ was administered after a 12-h fast and blood samples were collected in heparinized test tubes from the catheter after 1, 6, 24, 36, 48 and 72 h. The catheter was flushed with heparin in saline (100 units/ml) after each sample. Plasma was separated and all samples were stored at −20°C before analysis.

3. Results and discussion

3.1. Optimization of HPLC conditions

In the preliminary studies, separation of the diastereomers was performed under reversed-phase conditions. Although good resolution was observed, additional late eluting peaks were present. The

identity of these peaks, however, was unknown but may be attributed to the dimers or trimers of NEIC as reported by Wilson and Walle [14] for phenylethylisocyanate. This problem was overcome by using normal-phase HPLC system. Good baseline resolution of MFQ diastereomers ($\alpha > 1.3$) (Fig. 2) was achieved under the chromatographic conditions described in Section 2. The retention times of the NEIC derivatives of (+)-(*RS*)- and (−)-(*SR*)-MFQ and the I.S. were 16.4, 20.8, 8.1 and 10.6 min, respectively. No interfering peaks of endogenous substances and by-products were found in blank plasma, blood and urine. The exact order of elution of the peaks was determined using pure (−)-(*SR*)-MFQ and (+)-(*RS*)-MFQ. It was demonstrated that the (+)-(*RS*)-MFQ isomer eluted first.

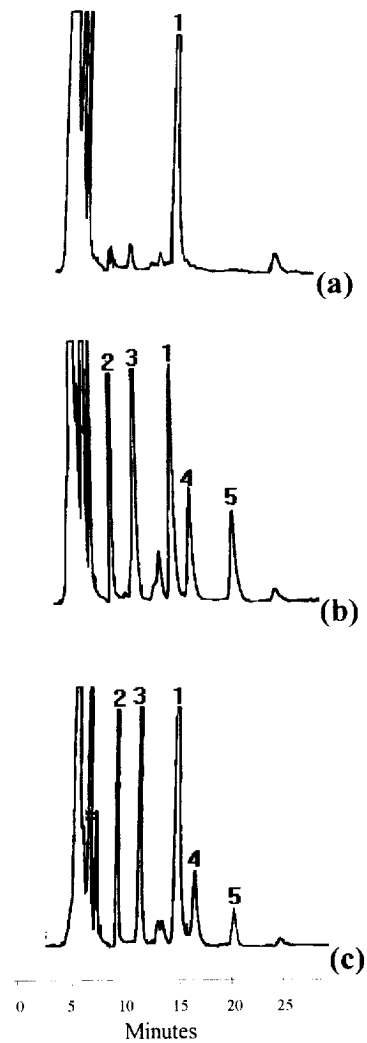


Fig. 2. HPLC chromatograms of MFQ and I.S. diastereomers. (a) Drug-free plasma; (b) plasma sample spiked with MFQ enantiomers (1 μg/ml of each enantiomer) and I.S.; (c) rat plasma sample, 6 h after oral administration of 50 mg/kg *rac*-MFQ. Peaks: 1, NEIC by-product; 2 and 3, I.S. diastereomers; 4, (+)-(*RS*)-MFQ diastereomer; 5, (−)-(*SR*)-MFQ diastereomer.

3.2. Derivatization reaction

Diastereomeric derivatives of MFQ were prepared by reaction with (+)-(*S*)-NEIC. This reagent, commercially available as a pure compound, increases UV and fluorimetric detection owing to its naphthyl moiety and has been used for determination of several drugs with amino or alcoholic groups [15–20].

The influence of the reagent concentration and the reaction temperature were studied using different molar excesses of the reagent. It was shown that by increasing the amount of the reagent, the peak area of the diastereomers was increased. The best result was achieved by a 120:1 molar excess of the reagent relative to MFQ. Larger amounts of the reagent lead to increased proportion of interfering by-products. The reaction seemed stable within 20 min both at room temperature and 45°C. The derivatization yield was 95.97±0.88 after 20 min at room temperature according to the method described in Section 2.6. For the purpose of structure elucidation, the diastereomers were isolated after several injections to the analytical column and combining the appropriate fraction using a fraction collector (Advantec, SF-2120, Dublin, CA, USA) connected to the chromatographic system. The diastereomers were relatively stable, and no changes were observed within a few days after repeated injection of the samples.

The UV absorption spectra of the diastereomers diluted in the mobile phase were obtained at room temperature using a UV-Vis Spectrophotometer (Model 160 Shimadzu, Kyoto, Japan). Two superimposable spectra were obtained with two maxima at 235 and 282 nm. The latter was used as the HPLC analytical wavelength.

An A.E.I. MS9 mass spectrometer (Manchester, UK) was used to take the fast atom bombardment mass (FAB-MS) spectrum of the diastereomers. The

medium was *m*-nitrobenzylalcohol on a copper probe tip using xenon bombardment at 8 keV. Both diastereomers showed identical fragmentation and molecular ion of *m/z* 576 $(M+H)^+$. The spectrum (Fig. 3) was consistent with a urea derivative. The loss of the derivatizing group gave *m/z* 379 (fragmentation 1) and *m/z* 198 (fragmentation 3) corresponding to the mefloquine and naphthylethylisocyanate. Other characteristic fragments were *m/z* 281 (fragmentation 2), *m/z* 170 (fragmentation 4) and *m/z* 155 (fragmentation 5). Fragment 2 was the one proving the binding of NEIC to piperidyl nitrogen as reported for other amino alcohol compounds at room temperature [15–19].

The presence of water in the reaction mixture resulted in an interfering peak eluted just before the first diastereomer. After separation of this fraction and obtaining the mass and NMR data, its structure was identified. Such a structure has been reported by Bjorkqvist and Toivonen using phenylisocyanate as reagent [21]. In order to overcome this problem the extracted organic layer was completely dried before derivatization and the reaction mixture was evaporated under nitrogen.

3.3. Extraction

Different extraction procedures [5,22–24] have been used to accomplish extraction of MFQ without any effect on derivatization. Most reported methods lead to interfering peaks after derivatization. The highest recovery with minimum interference was achieved by the extraction method described in Section 2.

The recovery of different biological samples of (+)-(*RS*)-MFQ and (−)-(*SR*)-MFQ enantiomers was 76–99% (Table 1). The recovery of the I.S. diastereomers was about 100% and the second peak was chosen for peak-area ratio calculations.

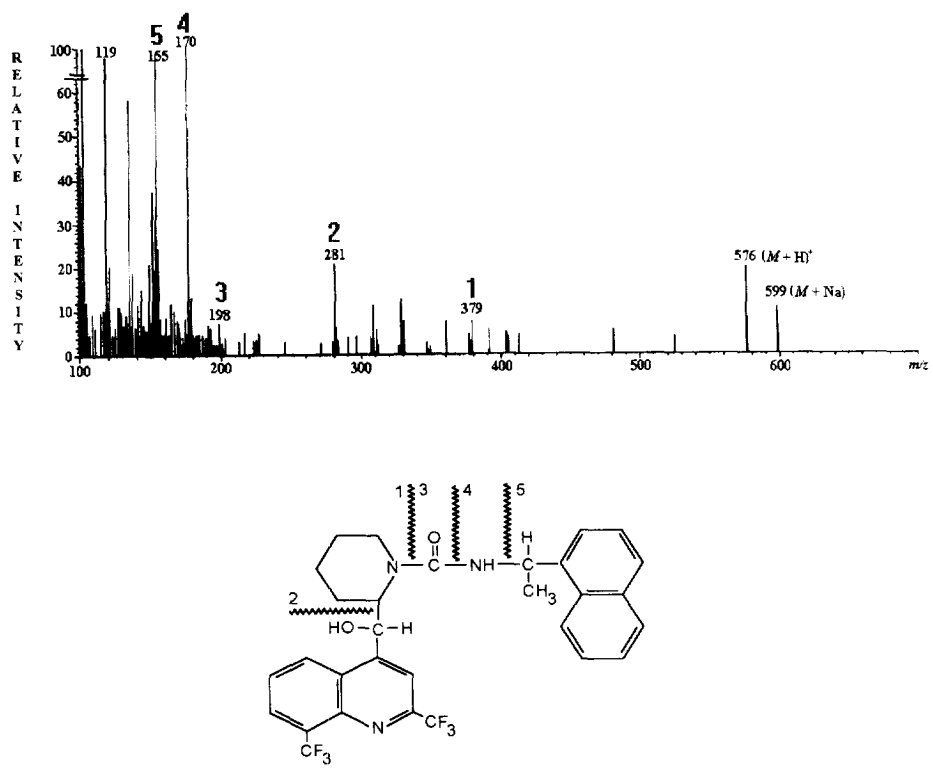


Fig. 3. MFQ–NEIC derivative structure and mass spectrum: numbers from 1 to 5 indicate the sites of fragmentation.

Table 1
Recovery of mefloquine enantiomers for plasma, blood and urine

Biological material	Concentration added (μg/ml)	% Recovery (mean±S.D.)	
		(+)-Mefloquine	(−)-Mefloquine
Plasma	0.20	98.79±1.44	83.49±1.21
	1.50	99.30±1.29	94.24±1.58
Blood	0.20	83.56±4.49	79.65±7.03
	1.50	75.91±1.25	78.55±3.16
Urine	0.08	86.98±2.98	88.64±1.19
	0.60	98.34±3.83	91.55±1.44

3.4. Calibration

A standard curve for MFQ enantiomers in different concentrations (0.2–1.5 μg/ml) in plasma and whole blood was prepared by addition of 50 μl of solutions 2–6 and 50 μl of I.S. solution to 200 μl drug-free samples. The same amounts of solutions 2–6 and I.S. were added to 500 μl drug-free urine to reach the concentration range of 0.08–0.6 μg/ml. The calibration curves displayed excellent linearity (r^2>0.998) in all biological samples. Typical calibration curves obtained for (+)-(*RS*)- and (−)-(*SR*)-MFQ in plasma described by $y=0.43x-0.022$ and $y=0.37x-0.02$, respectively. Calibration curves for (+)-(*RS*)- and (−)-(*SR*)-MFQ in whole blood were described by $y=0.46x-0.02$ and $y=0.47x-0.02$. Calibration curves for (+)-(*RS*)- and (−)-(*SR*)-MFQ in urine were described by $y=1.49x-0.02$ and $y=1.52x-0.02$. In all equations y is the peak–area ratio (MFQ/I.S.) and x is the enantiomer concentration.

3.5. Accuracy and precision

The accuracy and precision were determined by preparing three replicate samples of MFQ at concentrations of 0.2, 0.5 and 1.5 μg/ml of each enantiomer on three separate days. Concentrations were determined using a calibration standard curve prepared from the *rac*- MFQ in the range of 0.2–1.5 μg/ml of each enantiomer for each group of samples. Good reproducibility was observed over the entire concentration range. The results are presented in Table 2. The limit of determination of the assay, defined as the minimum concentration that could be

Table 2
Accuracy and precision in spiked plasma, blood and urine (n=9; three sets for 3 days)

Biological material	Concentration added (μg/ml)	(+)-Mefloquine			(−)-Mefloquine		
		Calculated (mean±S.D.)	%C.V.	%Error	Calculated (mean±S.D.)	%C.V.	%Error
Plasma	0.20	0.20±0.01	5.50	2.31	0.20±0.01	7.00	−1.44
	0.50	0.50±0.02	4.75	−0.24	0.50±0.02	3.85	0.34
	1.50	1.50±0.02	1.58	0.10	1.49±0.02	2.04	−0.50
Blood	0.20	0.19±0.01	6.33	−3.89	0.19±0.01	7.78	−2.76
	0.50	0.50±0.02	5.10	0.30	0.51±0.02	4.03	2.30
	1.50	1.50±0.05	3.54	−0.09	1.50±0.05	3.08	−0.01
Urine	0.08	0.08±0.00	4.55	−0.05	0.08±0.01	8.08	−0.97
	0.20	0.19±0.01	5.78	−2.32	0.20±0.01	7.40	−0.04
	0.60	0.59±0.02	4.41	−2.05	0.59±0.03	4.65	−2.11

measured with a C.V. better than 8%, was found to be 0.2 μg/ml for both enantiomers in plasma and whole blood and 0.08 μg/ml for urine samples. The limit of detection with a *S/N* ratio of 3:1 was 0.04 μg/ml in plasma and blood samples.

3.6. Application of the method

The plasma and whole blood concentration time profiles of (+)-(*RS*)-MFQ and (−)-(*SR*)-MFQ after oral administration of *rac*-MFQ to a male rat are shown in Fig. 4. The blood and plasma concentrations of (+)-(*RS*)-MFQ, were greater than (−)-(*SR*)-MFQ at most measured times. These results are in contrast with those reported for humans [5–7]. This might be a reverse stereoselectivity in rats which must be studied further.

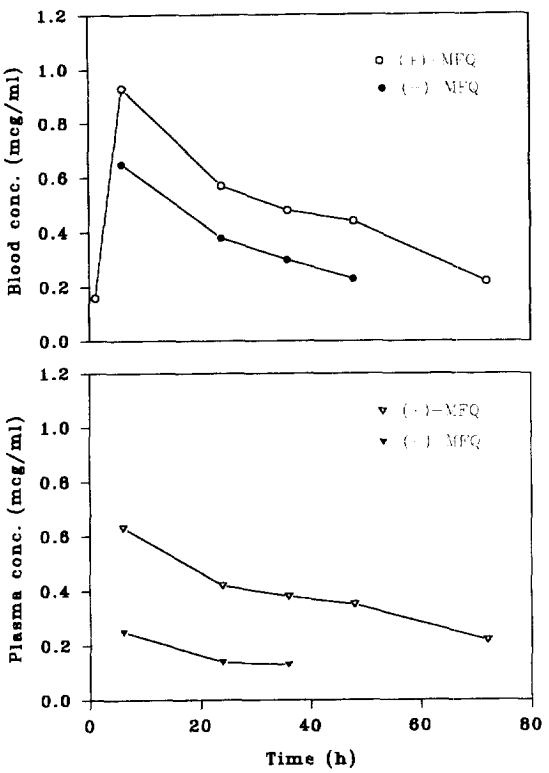


Fig. 4. Blood and plasma concentrations of mefloquine (MFQ) enantiomers in rat following a single oral dose of 50 mg/kg *rac*-MFQ.

4. Conclusion

The present method is comparatively rapid, simple, reliable and sensitive, allowing the processing of multiple determination in a short time. Using a commercially available reagent and separation of the diastereomers by normal-phase chromatography and UV detection with a low limit of determination in low volume of samples makes this method very suitable for pharmacokinetic studies.

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