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Enantioselective high-performance liquid chromatographic determination of (*SR*)- and (*RS*)-mefloquine in plasma using N-benzyloxycarbonyl-glycyl-L-proline as chiral counter ion

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ABSTRACT

A stereoselective HPLC method is described for the determination of (*SR*)- and (*RS*)-mefloquine in plasma. The direct chiral separation is carried out on a Hypercarb-S column (porous graphitised carbon) with N-benzyloxycarbonyl-glycyl-L-proline (L-ZGP) as a chiral counter-ion in a reversed-phase system. The sample work-up included protein precipitation by addition of zinc sulphate and acetonitrile followed by liquid–liquid extraction with methyl-*tert.*-butyl ether. After evaporation of the organic phase, the residue is dissolved in the mobile phase and injected onto the column. Analyses of the enantiomers in plasma after a single oral dose of mefloquine indicates that the pharmacokinetics of the two enantiomers are different. The method is validated by determining the absolute recovery, linearity, accuracy, precision and inter- and intra-assay variation.

INTRODUCTION

Mefloquine (MQ), D,L-erythro-α-(2-piperidyl)-2,8-bis-(trifluoromethyl)-4-quinolinemethanol is used for treatment and prophylaxis of malaria. Mefloquine is marketed as a racemic mixture of the erythro form of (−)(*SR*)-MQ (11S, 12R) and (+)(*RS*)-MQ (11R, 12S) (Fig. 1).

The enantiomers of MQ were not significantly different in their relative antimalarial activity against *Plasmodium berghei* in rodents [1]. Similar observations have been reported for human *Plasmodium falciparum in vitro* [2].

The enantiomers of the more commonly used antimalarial drug chloroquine have been shown to exhibit a stereoselective difference in their pharmacological activity [3]. Although few studies on the pharmacokinetics and metabolism of MQ enantiomers in humans have been reported, Gimenez *et al.* [4] have shown that after oral administration of racemic MQ, the plasma concentration of (*SR*)-MQ was significantly higher than that of (*RS*)-MQ.

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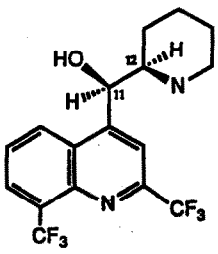
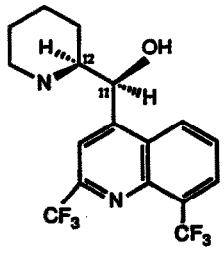


(+)-Mefloquine 11R, 12S



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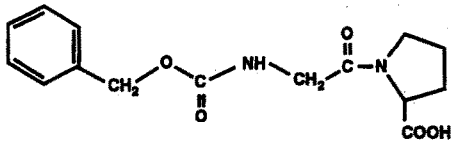

N-Benzyloxycarbonyl-glycyl-*L*-proline (*L*-ZGP)

Fig. 1. Structures of mefloquine (MQ) enantiomers and N-benzyloxycarbonyl-glycyl-L-proline (L-ZGP).

Assays have been described for the determination of racemic MQ in plasma by high-performance liquid chromatography (HPLC) [5–8] and by gas chromatography [9,10]. The side effects reported for MQ [11,12] could be related to the separate enantiomers. (*SR*)-MQ is a more potent inhibitor of acetyl- and butyryl-cholinesterase than (*RS*)-MQ [13]. Thus it is of great importance to be able to determine the enantiomers of MQ.

The determination of MQ enantiomers in plasma has previously been obtained by using a column-switching technique [4]. The coupled-column system consisted of an achiral cyano-bonded phase, an (*S*)-napthylurea chiral stationary phase and a silica pre-column. The silica column was used to concentrate the eluent from the achiral column using a backflushing technique. This method had a limit of determination of 0.1 μg/ml (0.25 μmol/l) for each enantiomer. A direct enantioselective method with α_1-acid-glycoprotein immobilised on silica as the chiral stationary phase was recently presented [14]. The limit of determination was 0.5 μmol/l for each enantiomer of MQ when using 500 μl plasma.

In this study the chiral counter-ion L-ZGP, a N-protected dipeptide, is used to promote enantioselective retention of MQ. The diastereomeric ion-pairs of L-ZGP and MQ were retained on the achiral solid phase (Hypercarb-S). Previously, L-ZGP has been used to resolve chiral amines of pharmacological interest, *e.g.* β-adrenergic blocking agents and for bioanalysis of propanolol enantiomers [15,16]

This paper presents a simple and selective chromatographic system for direct separation of MQ enantiomers. The method has a limit of determination of 0.5 μmol/l for each enantiomer when using 300 μl plasma. The method is suitable for pharmacokinetic studies.

EXPERIMENTAL

Chemicals

Racemic MQ and (*SR*)- and (*RS*)-MQ were gifts from Hoffman-La Roche, and the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (Washington, DC, USA). The enantiomers of MQ were prepared according to Carrol and Blackwell [1].

The chiral counter-ion L-ZGP (Fig. 1) was obtained from Nova Biochem, (Switzerland). The internal standard quinine (I.S.) was obtained from Apoteksbolaget (Sweden). HPLC-grade acetonitrile and methyl-*tert.*-butyl ether (MtBE) were obtained from E. Merck (Darmstadt, Germany). All other reagents and chemicals used were of analytical grade.

Chromatography

The chromatographic system consisted of a HPLC pump, Model 2150 (LKB-Pharmacia, Uppsala, Sweden) and the sample was injected by use of an auto-injector, Waters WISP 710B, (Waters, Milford, MA, USA). Separations were carried out using a porous graphitised-carbon column, Hypercarb-S (100 × 4.6 mm I.D., 7 μm) from Shandon Scientific (Cheshire, UK).

The mobile phase consisted of acetonitrile–methanol–acetate buffer, 0.06 mol/l, pH 4.6 (48:20:32, v/v/v) and L-ZGP 5.0 mmol/l. The pH of the mobile phase was adjusted to 4.6 with sodi-

um hydroxide. The system was operated at ambient temperature with a flow-rate of 0.8 ml/min.

The eluent was monitored at 278 nm with a Kratos Spectroflow 757 detector (Ramsey, NJ, USA). The chromatograms were evaluated with a Model SP 4270 integrator (Spectra Physics, San Jose, CA, USA).

Calibration and quantitation

Stock calibration solutions (100 μmol/l) of racemic MQ, (*SR*)- and (*RS*)-MQ were prepared in HCl 0.01 mol/l. Standard curves for (*SR*)- and (*RS*)-MQ were prepared by adding racemic MQ to one pool of drug-free plasma at concentrations ranging from 0.5 to 4.0 μmol/l. (*SR*)- and (*RS*)-MQ respectively, were added to two other pools at the same concentrations. The peak-height ratios from (*SR*)- and (*RS*)-MQ relative to the I.S. (quinine) were calculated and plotted *versus* the concentrations. The calibration curve was determined using the least squares regression line.

Patient sample and sample storage

Human patient plasma samples were collected at various intervals, using Vacutainer heparinised tubes, from one healthy male volunteer who had taken a 500-mg oral dose of MQ. The spiked plasma standards and all plasma samples taken from the patient were stored at −20°C prior to analysis. MQ was stable under these conditions [10].

Assay procedure

Plasma samples (300 μl) were added to polypropylene tubes. The sample was deproteinized by the addition of 75 μl of 0.1 mol/l zinc sulphate, followed by 750 μl of acetonitrile containing I.S. (3 mmol/l). The solution was thoroughly vortex-mixed for 15 s after the addition of each compound.

After standing for 15 min, the tubes were centrifuged at 10 000 *g* for 10 min. The supernatant was decanted into polypropylene tubes and 2.0 ml of 0.1% ammonium solution and then 6.0 ml MtBE were added to each tube. The samples were extracted for 30 min and centrifuged at 3000 *g* for 5 min. The upper organic layer was transferred to a conical polypropylene tube and evaporated to dryness at 80°C under a stream of air. The residue was reconstituted in 150 μl of mobile phase, and 100 μl were injected onto the HPLC system.

Accuracy, intra- and inter-assay

The accuracy, intra- and inter-assay precision were determined by analysing five drug-free pooled plasma samples to which enantiomeric MQ at concentrations of 0.25, 0.5, 1.0 and 2.0 μmol/l had been added. Four replicate samples from each concentration were analysed on four separate days. Concentrations were determined using a calibration standard curve prepared from the racemic MQ on the day of analysis. From the data obtained, the intra- and inter-assay precision were calculated.

Absolute recovery

The absolute recoveries of MQ enantiomers in human plasma after protein precipitation and extraction were determined by comparing the peak heights with those obtained from injection of the separate enantiomers dissolved in the mobile phase.

RESULTS AND DISCUSSION

Optimization of the chiral separation

Enantioselective retention in chiral ion-pair chromatography can be obtained by stereoselective ion-pair formation in the mobile phase and/or by selective distribution of the formed diastereomeric ion-pairs to a achiral stationary phase [17]. The separation of MQ enantiomers was carried out on a graphitised-carbon HPLC column as the adsorbing phase. Fig. 2 shows the effect of acetonitrile and methanol concentration of the mobile phase on the resolution (R_s) of the enantiomer and system peaks as well as the I.S. peak. The system peak is an extra peak that is characteristic for each chromatographic system and it occurs when distortion of the equilibria at the top of the column takes place after injection of the sample [18]. By changing the concentration of methanol and acetonitrile the retention times

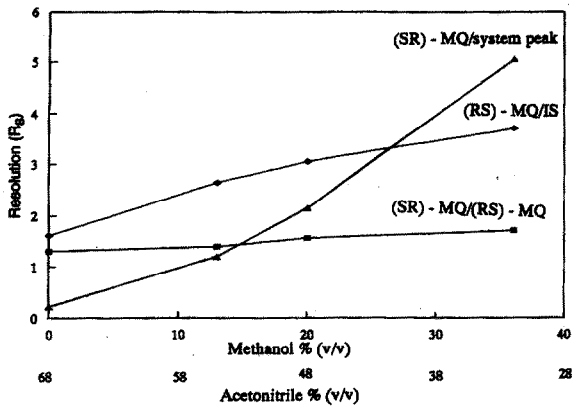

Fig. 2. Effect of the organic solvent concentration in the mobile phase on the resolution. Mobile phase: mixture of acetonitrile and methanol and constant amount of acetate buffer 0.06 mol/l, pH 4.6 and L-ZGP 5 mmol/l. Solid phase: Hypercarb-S.

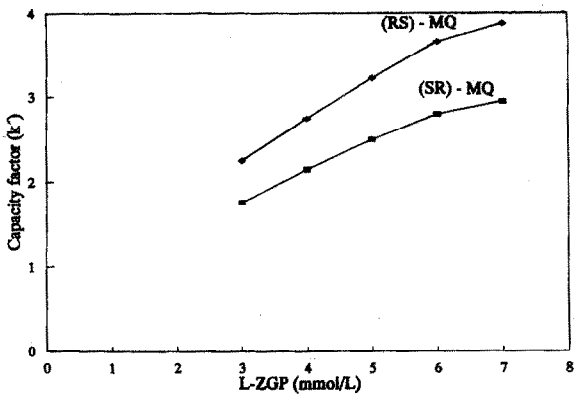

Fig. 3. Effect of L-ZGP concentration on the capacity factors (k') for MQ enantiomers. Mobile phase: acetonitrile–acetate buffer 0.06 mol/l, (60:40, v/v). Solid phase: Hypercarb-S.

of the system peak and the sample peaks was influenced, and hence the resolution changed. The resolution depends on the retention of the system peak and will be changed by the content of acetonitrile and methanol, since many additives in the mobile phase may give positive or even negative peaks [19,20]. The results in Table I show that the capacity factor, the resolution efficiency and the asymmetry factor are highly affected by the pH of the mobile phase. The influence of the L-ZGP concentration on the capacity factor (k') is given in Fig. 3. The increased retention of the enantiomers at high L-ZGP concentration is due to the more extensive formation of ion-pairs between the enantiomers and L-ZGP [17]. Based on these results, the optimum mobile-phase concentrations were determined to be acetonitrile–methanol–acetate buffer 0.06 mol/l, pH 4.6 (48:20:32, v/v/v), and L-ZGP 5 mmol/l. Baseline resolution of MQ enantiomers and patient samples respectively can be obtained as shown in Fig. 4. No interference from plasma components or the common antimalarial drug chloroquine was observed.

The elution order of the enantiomers was confirmed by separate injection of the pure (*SR*)- and

TABLE I

INFLUENCE OF APPARENT pH ON RETENTION, STEREO-SELECTIVITY, RESOLUTION, EFFICIENCY AND ASYMMETRY FACTOR

Solid phase: Hypercarb-S. Mobile phase: acetonitrile–methanol–acetate buffer 0.06 mol/l (48:20:32, v/v/v). L-ZGP 5 mmol/l.

pH	k'_{SR}	k'_{RS}	α	R_s	$H(\mu)_{SR}$ [a]	$H(\mu)_{RS}$ [a]	Asf_{RS} [b]	Asf_{SR} [b]
3.5	0.22	0.38	1.73	0.77	43	48	1.0	1.0
3.9	0.91	1.33	1.47	1.67	31	49	1.5	1.8
4.5	3.45	4.56	1.32	1.60	71	62	2.6	2.5
5.0	6.95	9.48	1.36	1.82	100	70	3.5	2.3
5.8	13.9	19.1	1.38	1.05	114	76	3.8	3.1

[a] H = plate height.

[b] Asymmetry factor at 10% of peak height.

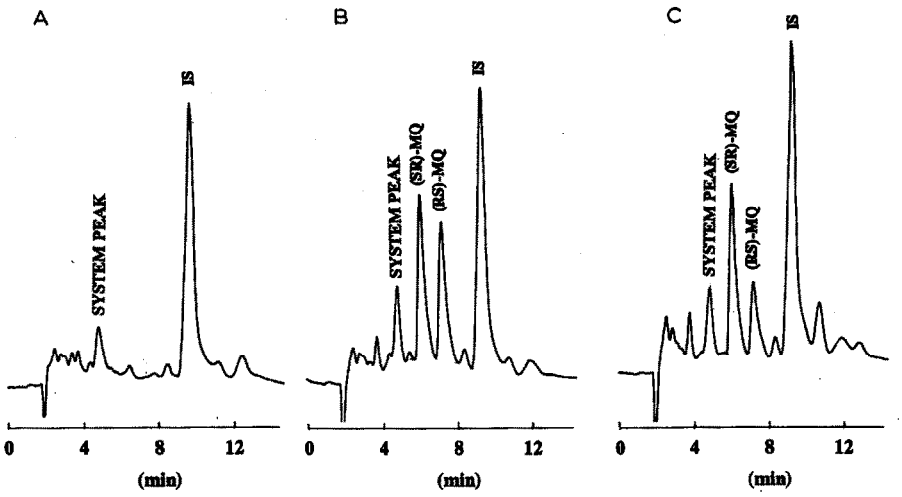

Fig. 4. HPLC chromatogram of separation of (*SR*)- and (*RS*)-MQ. (A) Drug free blank plasma; (B) spiked plasma with racemic MQ standard (2.0 μmol/l); (C) plasma sample obtained from a volunteer after an oral dose of 500 mg MQ. (*SR*)-MQ 0.94 μmol/l and (*RS*)-MQ 0.43 μmol/l.

(*RS*)-MQ. The elution order of the enantiomers can easily be reversed by exchange of L-ZGP for D-ZGP in order to facilitate the quantitation [21].

The stability and reproducibility of the chromatographic system

The stability of the chromatographic system was tested for a period of six weeks by a weekly analytical run and injection of 25 plasma samples (total 140 injection of extracts of plasma samples). The assay samples consisted of spiked plasma standards or patient samples analysed according to the assay procedure.

After each analytical run of plasma samples, the chromatographic system was washed with recirculation of 200 ml of methanol–acetic acid 0.2

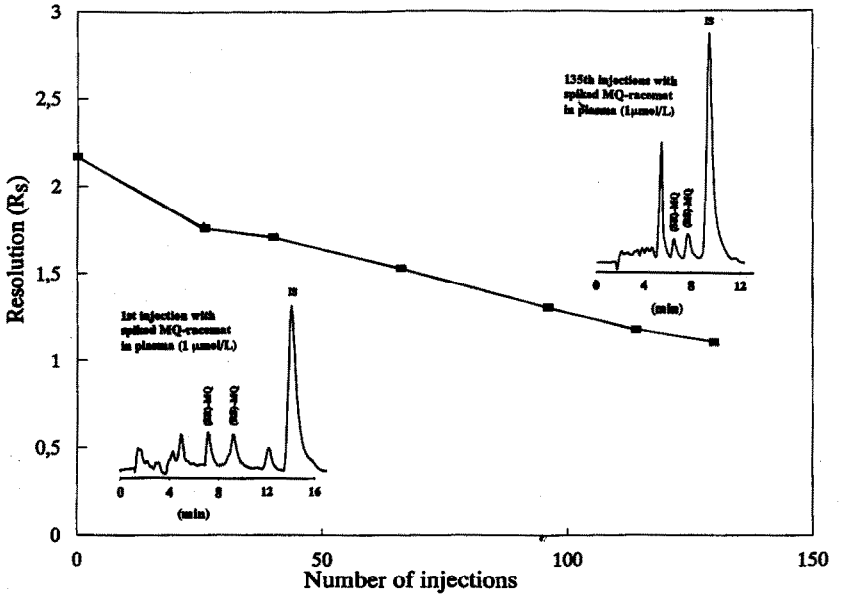

Fig. 5. Effect of the number of injections of plasma extract on the resolution (R_s) from the 1st injection to the 135th injection.

mol/l (95:5, v/v). Before a new analytical run with plasma samples, the column is equilibrated by recirculation of 700 ml of the mobile phase. New mobile phase (500 ml) was prepared before every new run and was recirculated during the assay.

Fig. 5 shows the resolution (R_s) of the 1st and 135th injection of plasma sample extracts. Even if the chromatographic system was washed after every run, there was a continuous decrease in resolution. This effect was probably due to adsorption of endogenous compounds to the carbon stationary phase, since no decrease in resolution was observed when injecting aqueous solutions of MQ.

Extraction

The protein precipitation procedure in the present assay for determination of the enantiomers in plasma was similar to that used for the determination of (*RS, SR*)-MQ [7]. The alkaline pH was achieved by the addition of 1% ammonia solution. Other buffer salts (hydroxide-, phosphate-, borate-) were found to give system peaks in the chromatogram that might interfere with the peaks of the analytes. Thus, the injected samples should be dissolved in a solvent similar to the mobile phase in order to avoid the occurrence of system peaks and deterioration of the peak efficiency. The absolute recoveries after the sample work-up procedure were higher than 72% for the enantiomers (Table II).

TABLE II

RECOVERY OF THE MQ ENANTIOMERS FROM SPIKED PLASMA (n = 8; 500 μl)

Added (μmol/l)	Recovery (mean ± S.D.) (%)
(SR)-MQ	
0.25	83.0 ± 4.2
0.5	72.9 ± 4.3
1.5	77.1 ± 2.7
3.0	76.8 ± 2.7
(RS)-MQ	
0.25	84.5 ± 2.1
0.5	83.1 ± 6.5
1.5	78.9 ± 2.8
3.0	76.9 ± 2.6

Calibration standard curve

The peak-height ratio calibration standard curves of the MQ enantiomers to I.S. (quinine) *versus* the concentration of each enantiomer were linear ($r > 0.999$) over the range 0.25–2.0 μmol/l. The slopes of the calibration curves were similar with a negligible y-intercept for both standards which were prepared from the racemate of MQ and from the separate enantiomers of MQ.

Correlation data

The sum of the enantiomer concentrations [(*SR*)- plus (*RS*)-MQ] (y) was compared with the concentration obtained from the achiral determination of (*SR, RS*)-MQ by an HPLC method [7] of the same plasma sample (x). The sum of the enantiomer concentrations was almost identical with the concentration determined by the achiral method ($y = 0.9469x + 0.0477$; $r = 0.9842$; $n = 23$).

Accuracy, inter- and intra assay precision

Table III shows the accuracy, inter- and intra assay precision data of the enantiomeric determination of MQ. The limit of determination for the method was 0.5 μmol/l for the separate enantiomers with an intra-assay variation of < 13% at 0.25 μmol/l.

Application of the method

The presented method was applied to the enantioselective determination of MQ in one healthy male. The plasma concentration–time profiles of (*SR*)-MQ, (*RS*)-MQ and racemic MQ after a 500-mg oral dose are shown in Fig. 6. The plasma concentration of (*SR*)-MQ was 2–3 times higher than that of (*RS*)-MQ. The same result was found by F. Gimenez *et al.* [4]. The reason for the higher plasma concentration of (*SR*)-MQ is unclear. It suggests a different bioavailability for the two enantiomers.

For routine analysis it would be advantageous to use the antipode, *i.e.* D-ZGP as counter-ion since the (*RS*)-MQ, which has a lower concen-

TABLE III

ACCURACY, INTRA- AND INTER-ASSAY PRECISION FOR MQ ENANTIOMERS FROM SPIKED PLASMA

Spiked concentration (pglmol)	Determined concentration (μmol/l)	*n*	C.V. (%)
Intra-assay: (SR)-MQ			
0.25	0.24	7	13
0.5	0.54	4	6.1
1.5	1.47	4	6.1
2.0	2.06	4	1.6
Intra-assay: (RS)-MQ			
0.25	0.25	7	9.4
0.5	0.46	4	5.2
1.5	1.40	4	6.9
2.0	2.02	4	3.9
Inter-assay: (SR)-MQ			
0.25	0.25	4	>30
0.5	0.53	4	9.9
1.5	1.43	4	4.9
2.0	2.05	4	1.6
Inter-assay: (RS)-MQ			
0.25	0.26	4	17
0.5	0.47	4	8.3
1.5	1.41	4	7.0
2.0	1.97	4	2.3

tration, would then elute before the main peak, *i.e.* the (*SR*)-MQ enantiomer [17]. Generally, the precision of the determination improves if the smaller peak is eluted before the larger peak.

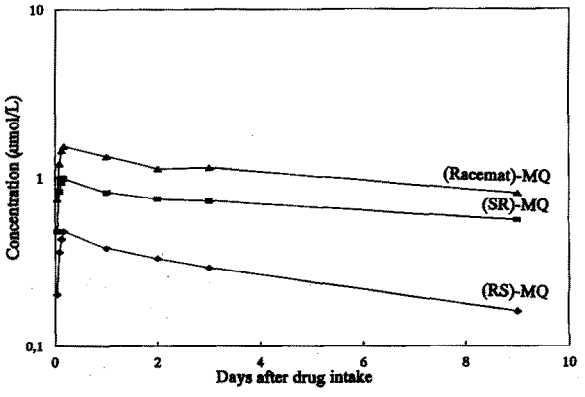

Fig. 6. Plasma concentration–time curve of racemate-MQ, (*SR*)-MQ and (*RS*)-MQ obtained from one healthy man after a single dose of 500 mg of racemic MQ.

CONCLUSION

Direct determination of the MQ enantiomers can be achieved with a chiral ion-pair HPLC method. The present method is validated by comparing the results obtained from the sum of (*SR*)- and (*RS*)-MQ enantiomers to that of (*SR, RS*)-MQ determined by the achiral method. This direct method is suitable for pharmacokinetic studies of the MQ enantiomers.

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