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High-performance liquid chromatographic determination of (*SR*)- and (*RS*)-enantiomers of mefloquine in plasma and capillary blood sampled on paper after derivatization with (−)-1-(9-fluorenyl)ethyl chloroformate

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Abstract

A sensitive, stereoselective and rapid reversed-phase liquid chromatographic method for the determination of (*SR*)- and (*RS*)-mefloquine enantiomers in 100 μl plasma and capillary blood collected on chromatographic paper is presented. The assay involves protein precipitation from plasma, liquid–liquid extraction of mefloquine from plasma, capillary blood with methyl *tert.*-butyl ether under alkaline conditions and derivatization of MQ with (−)-1-(9-fluorenyl)ethyl chloroformate. Liquid chromatographic separation of the diastereomers was performed using an C_{18} reversed-phase column with acetonitrile–water–acetic acid 82:18:0.07 (v/v/v) as the mobile phase, and a flow-rate of 1.0 ml/min. When using 100 μl of plasma the limit of determination is 250 nmol/l with ultraviolet- and 10 nmol/l with fluorescence detection. The present method offers several advantages over those previously reported; very low limit of determination, small sample volume, sampling onto paper and use of an inexpensive standard achiral HPLC column. No racemization during the derivatization procedure or storage of the MQ enantiomers was found.

1. Introduction

Mefloquine (MQ), D,L-erythro-α-(2-piperidyl)-2,8-bis-(trifluoro-methyl)-4-quinoline-methanol, is a drug used for malaria treatment and prophylaxis. Mefloquine has four stereoisomers, threo- and erythro-mefloquine, but the drug is marketed only in the erythro form as a racemic mixture of (−)(*SR*)- and (+)(*RS*)-MQ enantiomers (Fig. 1). The (*RS*)-enantiomer of MQ is 1.69 to 1.81 times more active than the (*SR*)-enantiomer against both a MQ-sensitive and a MQ-resistant *Plasmodium falciparum* strain *in vitro* [1]. The *SR*-enantiomer is a more active acetyl- and butyryl-cholinesterase inhibitor than the *RS*-enantiomer [2]. This indicates that the adverse reactions in humans and antimalarial activity might differ between the two enantiomers. Up to now there has been no pharmacodynamic and pharmacokinetic data available for MQ-enantiomers. This may be due to

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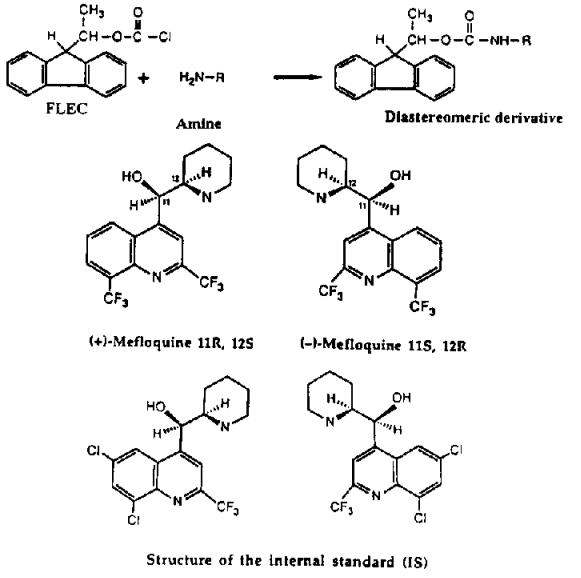

Fig. 1. Chemical structures of mefloquine, I.S. and (−)-1-(9-fluorenyl)ethyl chloroformate (FLEC) and the proposed reaction of FLEC with a primary amine to form a diastereomeric derivative.

the lack of a sensitive analytical method for the determination of the enantiomers.

Many analytical methods using HPLC techniques for measurement of MQ and its acid metabolite have been developed in the past five years having a limit of determination down to 0.1 μmol/l [3–5]. Gimenez *et al.* [6] developed an achiral–chiral HPLC method for the determination of MQ enantiomers by coupling a reversed-phase column and a column containing an (*S*)-naphtylurea chiral stationary phase. An enantioselective method using an α_1-acid glycoprotein chiral stationary phase was recently presented [7]. The limit of determination with the methods described above is 0.25–0.50 μmol/l using a 500-μl sample volume. For the determination of the enantiomeric purity of MQ commercial tablets, Qio *et al.* [8] presented an enantioselective HPLC method using a Chiralpak AD column for the separation of all four stereoisomers: (+)- and (−)-erythro and the (+)- and (−)-threo forms of MQ. The authors did not report any validation data for plasma samples.

In our laboratory we have recently developed a chiral method for the separation and determination of (*SR*)- and (*RS*)-MQ in plasma by chiral ion-pair chromatography on a Hypercarb-S carbon column [9]. However, this column was not stable enough for large numbers of plasma samples.

The aim of the present work was to develop an enantioselective HPLC method for the determination of (*SR*)- and (*RS*)-MQ enantiomers in biological fluids with a very low limit of determination using an inexpensive standard achiral column.

2. Experimental

2.1. Chemicals and reagents

The pure (*SR*)- and (*RS*)-enantiomers of MQ and the internal standard, D,L-erythro-α-(2-piperidyl) - 2 - trifluoromethyl - 6,8 - dichloro - 4 - quinolinemethanol (I.S.) (Fig. 1) were donated by the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (Washington, DC, USA). The MQ racemate was supplied by Roche (Skarholmen, Sweden) and the separate enantiomers of (+)-and (−)-1-(9-fluorenyl)ethyl chloroformate (FLEC) were a gift from Eka Nobel (Surte, Sweden). The purity of (+)-FLEC and (−)-FLEC was greater than 99.9%. All other reagents and chemicals were of analytical reagent grade.

2.2. Standard and derivatization reagent preparation

Stock standard solutions of MQ enantiomers and racemate were prepared by dissolving the salts in HCl 0.01 mol/l to a final concentration of 100 μmol/l. The solutions were stored at +4°C. Working standard solutions were obtained by diluting the stock solutions with either acetonitrile, drug free plasma or whole blood.

(−)-FLEC stock solution (10–20 mmol/l) was prepared from crystalline (−)-FLEC and dissolved in water free acetonitrile. It was stable in glass ampoules at +4°C for at least 10 months. On each day, (−)-FLEC and (+)-FLEC working solution were prepared by diluting the (−)-

FLEC and (+)-FLEC stock solutions with acetonitrile to 0.4 mmol/l.

2.3. Chromatography

The chromatographic (HPLC) system consisted of an SP-8810 HPLC pump (Spectra-Physics, San Jose, CA, USA). The sample was injected by using of a Waters WISP 710B autoinjector (Waters, Milford, MA, USA). Diastereomeric derivatives of MQ and I.S. were separated at ambient temperature utilizing a 250 × 4.6 mm I.D. Ultrasphere analytical column with 5-μm octadecylsilica packing material (Beckman, Irvine, CA, USA). The mobile phase consisted of acetonitrile–water–acetic acid (82:18:0.07, v/v/v) and was pumped at a flow-rate of 1.0 ml/min. The UV-detector was a Kratos Spectroflow 757 detector (Ramsey, NJ, USA) set at 263 nm, or a Shimadzu RF-551 fluorescence detector (Kyoto, Japan) with excitation at 263 nm and emission at 475 nm. The chromatograms were evaluated with a Model SP 4270 integrator (Spectra-Physics, San Jose, CA, USA)

2.4. Chromatographic paper

Whatman 31 ET Chroma chromatographic papers (Whatman, Maidstone, UK) were used.

2.5. Human samples

Vacutainer heparin tubes (Becton Dickinson, Rutherford, NJ, USA) were used for collection of plasma samples in two healthy volunteers after intake of a single dose of 250 or 500 mg of MQ as well as in travellers during standard MQ prophylaxis. From the Vacutainer tubes with whole blood, 100 μl were applied to chromatographic paper. All samples were stored at −20°C until analysis.

2.6. Plasma samples

Plasma samples (100 μl) were vortex-mixed for 15 s in polypropylene tubes after addition of 25 μl of 0.1 mol/l zinc sulphate solution. Following addition of 750 μl I.S. solution (1 μmol/l) in acetonitrile samples were again vortex-mixed for 15 s. After standing for 15 min the tubes were centrifuged at 10 000 *g* for 10 min. The supernatant was decanted into polypropylene tubes and 4.0 ml of borate buffer, 0.01 mol/l, pH 10.0 were added to each tube. The samples were then extracted with 5.0 ml methyl *tert.*-butyl ether (MtBE) by shaking for 20 min. The tubes were then centrifuged at 3000 *g* for 5 min. The upper organic layer was transferred to a conical polypropylene tube and evaporated to dryness at 65°C. After evaporation of the organic phase, 125 μl of (−)-FLEC working solution (0.4 mmol/l) were added and vortex-mixed, followed by addition of 50 μl of borate buffer (15 mmol/l, pH 8.5) and vortex-mixing. The sample was left at ambient temperature for 40 min, and after centrifugation at 3000 *g* for 10 min, 100 μl were injected onto the HPLC system. Either ultraviolet- or fluorescence detection was used.

2.7. Capillary blood dried on chromatographic paper

Papers with dried 100-μl blood spots (capillary whole blood samples from volunteers or whole blood standards) were cut into small pieces and transferred to polypropylene tubes. A 150-μl volume of I.S. (5 μmol/l dissolved in water) was added to each tube followed by 2.0 ml of ammonia–water (90:10, v/v). After incubation for 30–60 min at room temperature, the tubes were sonicated in an ultrasonic bath for 30 min at 37°C. The liquid–liquid extraction and derivatization were performed as described for plasma samples. A 100-μl volume was injected onto the HPLC system and fluorescence detection was used.

2.8. Recovery

The recovery was estimated against MQ enantiomers dissolved in acetonitrile. (−)-FLEC working solution and borate buffer were added to form the diastereomers, and the sample was injected onto the HPLC system. Peak heights were then compared with MQ enantiomers

added to plasma at three different concentrations (0.25, 0.5 and 1.5 μmol/l) after the sample had been processed according to the assay procedure. The recovery from spiked blood applied to paper was evaluated at 0.25, 0.5 and 1.0 μmol/l. Fluorescence detection was used.

2.9. Calibration procedure

Calibration graphs were prepared by analysis of five 100-μl drug-free plasma samples spiked with different concentrations of racemic MQ (0.25–2.0 μmol/l). The samples were extracted and derivatized as in the assay procedure. A calibration graph was obtained by plotting peak-heights ratio of (*SR*)- and (*RS*)-MQ to the I.S. against the concentration of the enantiomers. For consistency, the last elution peak of the I.S. enantiomers was always used for the peak-height ratio calculations. The calibration graphs for paper samples were obtained by adding racemate MQ standard to whole blood and applying 100 μl to chromatographic paper.

2.10. Accuracy, intra- and inter-assay precision

Plasma samples containing 0.050, 0.10, 0.25, and 1.5 μmol/l of MQ enantiomers were prepared by adding appropriate volumes of stock racemate standard solutions to drug-free plasma. The samples were stored at −20°C until analyzed. For paper samples, 100 μl whole blood spiked with MQ enantiomers at three different concentrations (0.125, 0.25, 0.50 μmol/l) were applied to chromatographic paper. Several samples from each concentration were analyzed on different days and concentrations were determined by comparison with a calibration graph prepared on the day of analysis. From the obtained data intra- and inter-assay precision was calculated.

2.11. Correlation data with other methods

The total concentration of the enantiomers (*SR*- plus *RS*-MQ) in plasma from 44 travellers during standard MQ prophylaxis was analyzed and compared with the racemate analysis of MQ in the same samples according to an HPLC method [5]. Finally, the *SR*- and *RS*-MQ concentrations were determined in 21 of the plasma samples from travellers with the present method and compared with the analysis performed with an other enantioselective HPLC method [9].

3. Results and discussion

FLEC was used for the first time as a chiral precolumn derivatizing reagent by Einarsson *et al.* [10] to separate (*R*)- and (*S*)-metoprolol. FLEC has also been used for the determination of propranolol [11] and atenolol [12] in plasma. A derivatizing reagent similar to FLEC, *e.g.* 9-fluorenylmethylchloroformate (FMOC), has been used for determination of racemic MQ from filter paper sampling [13].

3.1. Optimization of HPLC conditions

The addition of acetic acid to the mobile phase reduced the solvent front of underivatized (−)-FLEC when UV-detection was used. Chromatograms of the separation of (*SR*)- and (*RS*)-MQ and I.S. are shown in Fig. 2. The diastereomeric derivatives of (*SR*)- and (*RS*)-MQ are eluted before the derivatives of the I.S. The identity of the individual peaks was determined by assaying pure enantiomers of MQ and comparing the retention times. No interfering peaks of endogenous substances were found in human blank plasma or blank capillary blood, and no other commonly used antimalarial drugs (chloroquine, quinine, pyrimethamine, sulfadoxine) were detected either with UV- or fluorescence detection.

3.2. Derivatization reaction

The derivatization reaction (Fig. 1) yields stable diastereomers that can be detected by UV- or fluorescence detection. In order to optimize the derivatization reaction, the influence of (−)-FLEC concentration, pH and concentration of borate buffer, acetonitrile content in the reaction mixture and the reaction temperature was studied. It is necessary to use (−)-FLEC in a

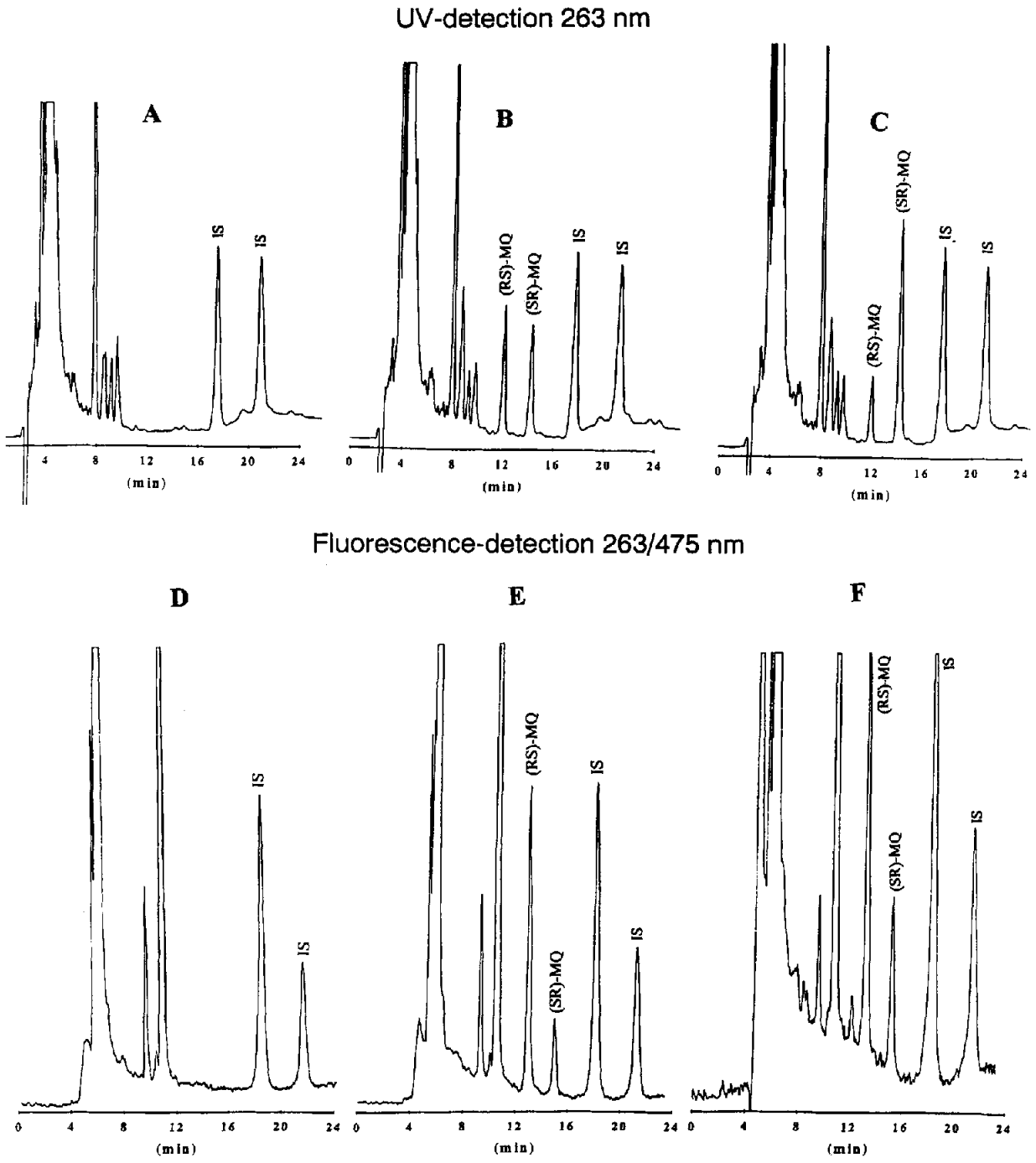

Fig. 2. HPLC chromatograms of the separation of (*SR*)- and (*RS*)-MQ and I.S. after extraction and derivatization with (−)-FLEC. A and D: Drug free plasma spiked with I.S. (5.0 μmol/l); B and E: Plasma spiked with racemic MQ standard (3.0 μmol/l) and with I.S. (5.0 μmol/l); C and F: Plasma samples obtained from a volunteer after an oral dose of 500 mg MQ. The plasma sample was taken 8 h after intake of the dose. (*SR*)-MQ: 3.16 μmol/l and (*RS*)-MQ 0.65 μmol/l. Chromatographic conditions as described in Experimental.

large molar excess (20:1 to 40:1) relative to MQ. The optimum of acetonitrile in the reaction mixture is 55–75% (v/v). A higher content of acetonitrile leads to precipitation of borate and a lower concentration leads to precipitation of (−)-FLEC and a decrease of derivatization yield. The final conditions for the derivatization were: acetonitrile 71% (v/v), borate buffer 4.3 mmol/l at pH 8.5, and (−)-FLEC concentration of 0.28 mmol/l.

It was found that the derivatization reaction proceeded faster at higher reaction temperatures, but this was of no practical value for this assay. The derivatization products of the enantiomers are stable at room temperature for at least 5 days. The fluorescence response of the derivatization product of the (*RS*)-MQ enantiomer is *ca.* four times as high as that of the (*SR*)-MQ derivative. For the I.S. the response of the first eluted peak is *ca.* two times as high if (−)-FLEC is used (Fig. 2E). The change in response can easily be reversed by using (+)-FLEC as a derivatization reagent.

3.3. Recovery

The recovery from plasma of (*SR*)- and (*RS*)-enantiomers was 76–81% at concentrations ranging from 0.25–1.5 μmol/l (Table 1) and from blood dried at chromatographic paper 55–60% at concentrations ranging from 0.25–1.0 μmol/l (Table 2). In the present method we used for the plasma sample the liquid–liquid extraction presented in ref. 5 and for capillary sampling on chromatographic paper that described in ref. 14: The efficiency of extraction with these methods was 80–90% and 70–80% respectively.

Table 1
Recovery for (*SR*)- and (*RS*)-MQ from spiked plasma ($n = 8$)

Sample concentration (μmol/l)	Recovery (mean ± S.D.)(μmol/l)
(SR)-MQ	
0.25	76.0 ± 8.6
0.50	80.7 ± 5.4
1.50	76.9 ± 6.4
(RS)-MQ	
0.25	79.6 ± 7.9
0.50	79.8 ± 6.1
1.50	78.6 ± 6.0

Table 2
Recovery for (*SR*)- and (*RS*)-MQ from spiked blood applied to chromatographic paper and dried ($n = 4$)

Sample concentration (μmol/l)	Recovery (%) (Mean ± S.D.)(μmol/l)
(SR)-MQ	
0.25	54.9 ± 4.5
0.50	58.1 ± 6.0
1.00	56.8 ± 6.9
(RS)-MQ	
0.25	59.5 ± 9.1
0.50	59.9 ± 7.4
1.00	57.5 ± 8.7

3.4. Accuracy, inter and intra-assay precision

The accuracy, intra- and inter-assay precision data for the determination of MQ enantiomers in plasma are presented in Table 3. The limit of determination was 0.25 μmol/l with UV- and 0.010 μmol/l for (*RS*)-MQ and 0.050 μmol/l for (*SR*)-MQ with fluorescence detection using 100 μl plasma at an inter-assay variation of less than 21%. For sampling on filter paper using a 100-μl sample, the limit of determination is 0.125 μmol/l (Table 4).

3.5. Correlation data

Fig. 3 shows the sum of (*SR*)- plus (*RS*)-MQ concentration determined by the method described above and the total MQ concentration obtained from the achiral determination of MQ according to the HPLC method [5]. The sum of the (*SR*)- and (*RS*)-MQ concentrations is very similar to the total concentration determined by the achiral method. Fig. 4 shows the good correlation between the present method and an other enantioselective HPLC-method [9] for

Table 3
Accuracy, intra- and inter-assay precision of (*SR*)- and (*RS*)-MQ added to plasma

	Added concentration (μmol/l)	Determined concentration (μmol/l)	Number of replicates	C.V. (%)
Intra-assay (SR)-MQ				
UV-detection	0.25	0.24	4	6.6
	1.50	1.47	5	4.2
Fluorescence-detection	0.050	0.059	6	11
	0.10	0.11	6	9.1
	0.25	0.26	6	9.6
Intra-assay (RS)-MQ				
UV-detection	0.25	0.27	4	8.0
	1.50	1.47	5	5.8
Fluorescence-detection	0.010	0.011	6	5.9
	0.050	0.054	6	5.0
	0.10	0.11	6	4.9
	0.25	0.25	6	5.7
Inter-assay (SR)-MQ				
UV-detection	0.25	0.26	7	15
	1.50	1.47	7	4.2
Fluorescence-detection	0.050	0.062	4	18
	0.10	0.10	4	14
	0.25	0.27	4	9.6
Inter-assay (RS)-MQ				
UV-detection	0.25	0.24	7	21
	1.50	1.47	7	5.7
Fluorescence-detection	0.010	0.013	4	16.2
	0.050	0.056	4	5.4
	0.10	0.11	4	5.4
	0.25	0.26	4	6.1

determination of (*SR*)- and (*RS*)-MQ enantiomers.

3.6. Application

Due to the high analytical sensitivity, both enantiomers could be determined in an adult 16 days after intake of 500 mg MQ from 100-μl dried spots of whole blood applied to chromatographic paper (Fig. 5). The (*SR*)-MQ concentration is *ca.* 2–5 times higher than the (*RS*)-MQ concentration in plasma from volunteers (Fig. 2C).

4. Conclusion

The present method has a lower limit of determination [10 nmol/l for (*RS*-MQ) and 50 nmol/l for (*SR*-MQ)] than other methods [7–9] and uses a lower plasma sample volume (100 μl) for the determination of (*SR*)- and (*RS*)-MQ. This makes it very suitable for pharmacokinetic studies. The method can also be used for determination of the enantiomers in capillary blood samples dried onto chromatographic paper. For sampling on filter paper using 100-μl blood samples the limit of determination is 0.25 μmol/

Table 4
Accuracy, intra- and inter-assay precision of (*SR*)- and (*RS*)-MQ added to whole blood and dried on to chromatographic paper

	Added concentration (μmol/l)	Determined concentration (μmol/l)	Number of replicates	C.V. (%)
Intra-assay (SR)-MQ				
Fluorescence-detection	0.125	0.127	6	12
	0.25	0.21	6	9.0
	0.50	0.44	6	4.5
Intra-assay (RS)-MQ				
Fluorescence-detection	0.125	0.108	6	5.8
	0.25	0.22	6	4.4
	0.50	0.48	6	6.9
Inter-assay (SR)-MQ				
Fluorescence-detection	0.125	0.119	3	21
	0.25	0.22	3	18
	0.50	0.45	3	5.4
Inter-assay (RS)-MQ				
Fluoresence-detection	0.125	0.113	3	7.9
	0.25	0.23	3	8.2
	0.50	0.46	3	7.3

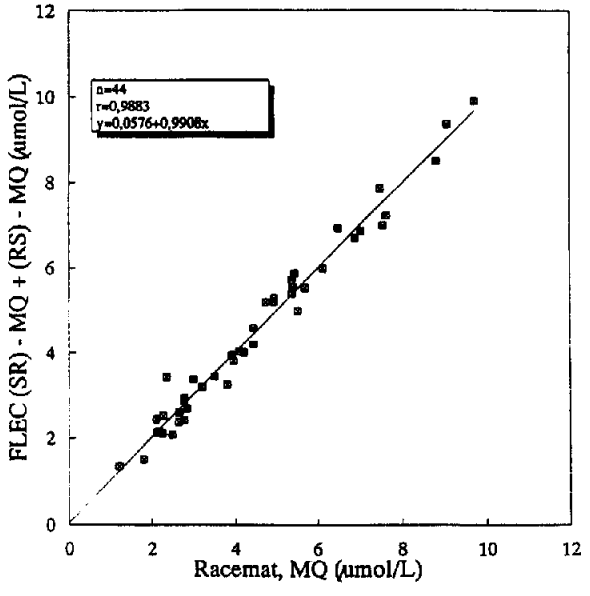

Fig. 3. Comparison of results for MQ concentration in plasma obtained by the HPLC method [5] as total concentration (x) and the sum of [(*SR*)- and (*RS*)-MQ] concentration (y).

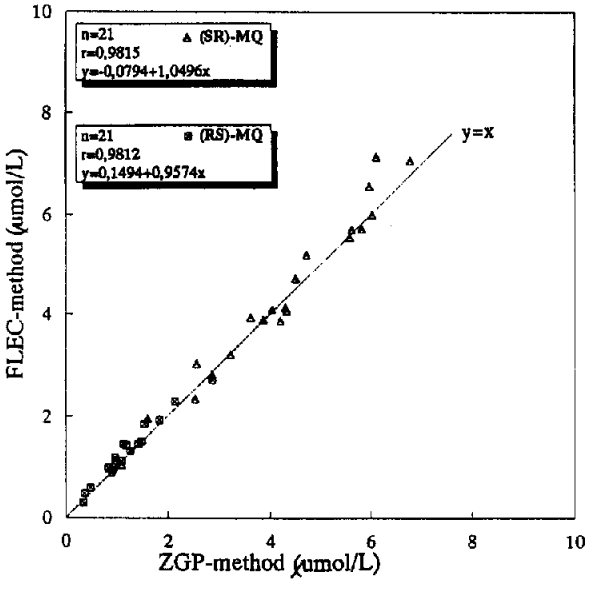

Fig. 4. Comparison of results for (*SR*)- (△) and (*RS*)-MQ (□) plasma concentration as determined by the present method and the enantioselective ZGP-method [9].

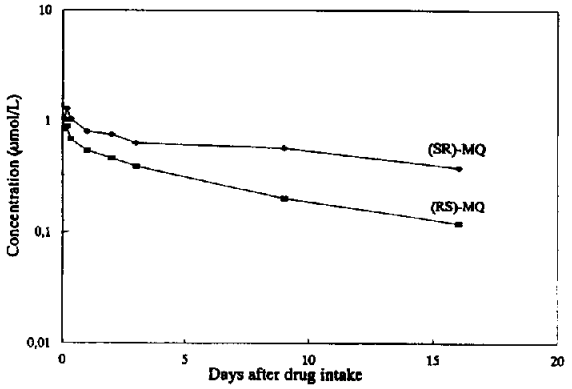

Fig. 5. (*SR*)-MQ and (*RS*)-MQ whole blood concentrations in 100-μl dried blood spots in one healthy volunteer after a single dose of 500 mg of racemic MQ.

l. The fluorescence detection increases the sensitivity fivefold over the UV detection at 263 nm.

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