Journal of Chromatography, 427 (1988) 295–305
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4142

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND EXTRACTION INVESTIGATION FOR THE SIMULTANEOUS DETERMINATION OF MEFLOQUINE AND ITS CARBOXYLIC ACID METABOLITE

YNGVE BERGQVIST*, SOLVEIG ECKERBOM and NIKLAS LARSSON

Department of Clinical Chemistry, Falun Central Hospital, S-79182 Falun (Sweden)

and

FREDERICK C. CHURCHILL

Division of Parasitic Diseases, Center for Infectious Diseases, Centers for Disease Control, Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA 30333 (U.S.A.)

(First received November 26th, 1987; revised manuscript received February 2nd, 1988)

SUMMARY

Distribution ratios of mefloquine ion pairs with perchlorate and heptanesulphonate as counter-ions, the base distribution for mefloquine, the acid distribution for the carboxylic acid metabolite and the ion-pair distribution with quaternary ammonium ions as counter ions have been determined. Differences in retention characteristics with several commercially available supports bearing the same octadecyl label were found. The effect on the retention time of the pH, the percentage acetonitrile and the addition of an amine to the mobile phase were studied. Other antimalarial drugs present concurrently in the sample showed no chromatographic interference with mefloquine and its metabolite. A suitable internal standard for use in the analytical method was also tested.

INTRODUCTION

Mefloquine (MQ) (Fig. 1) is a new antimalarial drug effective against chloroquine-resistant strains of *Plasmodium flaciparum*. MQ has been determined in biological samples by gas chromatography [1–3], liquid chromatography [4–6], and thin-layer chromatography [7]. Only two publications [6,7] describe the simultaneous determination of MQ and its main metabolite, 2,8-bis(trifluoromethyl)-4-quinoline carboxylic acid (MMQ) (Fig. 1) in biological samples. We found no information in these reports on the best organic solvent system for extracting MQ and MMQ from biological samples or on optimizing

0378-4347/88/$03.50

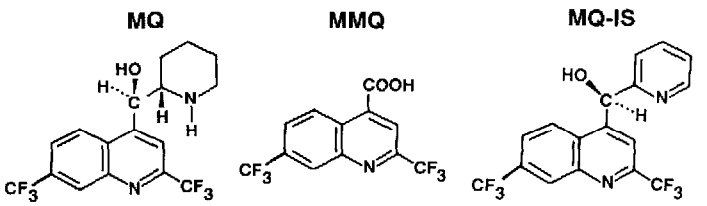

Fig. 1. Molecular structures of mefloquine (MQ), its metabolite (MMQ) and the internal standard (MQ-IS).

the chromatographic conditions. Because reliable information concerning the optimal conditions for extraction and chromatography is needed to construct an analytical method for simultaneous determination of MQ and MMQ in biological samples using a suitable internal standard, we performed the investigations described in this paper.

EXPERIMENTAL*

Apparatus

A Beckman DU-7 spectrophotometer (Beckman, Irvine, CA, U.S.A.) was used for UV spectrophotometric measurements. A Whatman PHA 250 digital pH meter (Whatman, Maidstone, U.K.) equipped with a combined glass electrode was used for pH measurements.

The chromatographic system included a Model SP8700XR HPLC pump (Spectra-Physics, San Jose, CA, U.S.A.), WISP 710B autoinjector (Waters Assoc., Milford, MA, U.S.A.), and a Spectroflow 757 (Kratos, Ramsey, NJ, U.S.A.) variable-wavelength UV detector set at 285 nm. Chromatographic parameters were evaluated using 175 mm × 4.6 mm I.D. stainless-steel columns packed in the laboratory by the upward slurry packing method with acetone as the suspension medium. Spherisorb ODS-1 5-μm packing material was used (Phase Separations, Queensferry, U.K.). Other commercially available columns were also tested (Table I). The flow-rate through the column, at ambient temperature, was 1 ml/min. The peaks were evaluated with a Model 4100 computing integrator (Spectra-Physics).

Chemicals and reagents

HPLC-grade acetonitrile and methyl *tert.*-butyl ethyer (MTBE) were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Diethyl ether (DEE) and methylene chloride (MDC) were of p.a. quality from Merck (Darmstadt, F.R.G.). Heptanesulphonic acid was obtained from Sigma (St. Louis, MO, U.S.A.). The quaternary ammonium compounds were chloride

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

TABLE I

STATIONARY PHASES

All particle sizes 5 μm; mobile phase, phosphate buffer (0.1 *M*, pH 2.50)–acetonitrile (50:50, v/v); flow-rate 1.0 ml/min.

Manufacturer	Name	Dimensions	Retention time (min)		
			MQ	MMQ	MQ-IS
Phase Separations*	Spherisorb ODS-1	175 mm×4.6 mm I.D.	6.0	7.6	8.5
Phase Separations*	Spherisorb ODS-2	175 mm×4.6 mm I.D.	5.9	11.0	11.6
Merck	LiChrosorb RP-18	250 mm×4.5 mm I.D.	5.7	10.2	9.6
Macherey-Nagel	Nucleosil C_{18}	150 mm×4.9 mm I.D.	3.4	9.6	9.2
Beckman Instruments	Ultrasphere XL-ODS	70 mm×4.6 mm I.D.	2.0	6.5	2.2
Supelco	LC-18DB	250 mm×4.0 mm I.D.	6.6	18.0	14.0

*Packed in the laboratory.

salts from Sigma. N,N-Dimethyloctylamine (DMOA) was obtained from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals were of analytical quality.

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).

Determining distribution ratios

The distribution ratios (D_A) between phosphate buffers (0.3 *M*) of pH 3.00 and 9.00 with various counter ions (0.05–0.1 *M*) and three organic solvents were determined for MQ, MMQ and MQ-IS. An ion-pair extraction technique was evaluated that effected simultaneous extraction of MQ and MQ-IS, which are bases, together with the acid metabolite MMQ, either at pH 3.00 or at pH 9.00. An inorganic anionic counter ion, perchlorate, and an organic anionic counter ion, heptanesulphonate, were evaluated for the extraction of MQ at pH 3.0. Three different quaternary ammonium counter ions were evaluated for the extraction of MMQ at pH 9.00. All extraction experiments were carried out in duplicate in two different series (Table II), with equal volumes (6 ml) of aqueous and organic phase, and at 25 °C (equilibration time 30 min). The concentrations of the drugs in the organic phase were calculated as the difference between the initial concentration (50–75 m*M*) in the aqueous phase and the concentration in the aqueous phase found at equilibrium. Concentrations were determined by photometric measurements at 285 nm.

If equal phase volumes of aqueous and organic phases are used, the percentage recovery (P) of the drugs in the organic phase can be calculated by the following formula [8]:

$$P=\frac{1}{1+1/D_A}\times 100\%.$$

Solvent for the residue

Solutions containing equal concentrations (10 μM) of MQ and MMQ in phosphate buffer (0.1 M, pH 6) and acetonitrile at various concentrations were prepared and chromatographed with different injection volumes (25, 50 and 100 μl). A Spherisorb ODS-1 column (5 μm) was used, with acetonitrile–phosphate buffer (0.1 M, pH 2.50) (50:50, v/v).

RESULTS AND DISCUSSION

Extraction properties

A theoretical elucidation of the extraction, including the side-reactions, was not done in this study. The distribution ratio D_A is defined by:

$$D_A = C_{A,org} \times (C_{A,aq})^{-1}$$

where $C_{A,org}$ and $C_{A,aq}$ are the total concentrations of the substance A in the organic and aqueous phase, respectively (Table II). Different combinations of organic solvents and the ion-pairing agent could be used to achieve an extraction recovery of greater than 97% from an aqueous phase at pH 3.00 for all the drugs. Heptanesulphonate confers a more hydrophobic nature on the resulting ion pair than does perchlorate. At a concentration of 50 mM of both ion-pairing anions, the recovery of MQ was greater than 97% in the organic phase. The distribution ratios for the drugs were constant when the pH of the phosphate buffer was between 2.0 and 4.0.

MMQ could be extracted at pH 9.00 into DEE organic phase with tetrahexyl-

TABLE II

DISTRIBUTION RATIOS (LOGARITHMIC) OF MQ, MMQ AND MQ-IS BETWEEN DIFFERENT ORGANIC SOLVENTS AND AQUEOUS PHASES

All data are means from two series with duplicate extraction experiments. Extraction recovery in parentheses.

Substances	pK_a	Organic phase	Aqueous phase*			
			pH 3.0 (without counter ion)	pH 3.0 (perchlorate, 0.05 M)	pH 3.0 (heptane-sulphonate, 0.05 M)	pH 9 (without counter ion)
MQ	8.5	DEE	−1.1 (7.4)	1.8 (98.4)	–	1.8 (98.4)
		MDC	−1.5 (3.1)	2.0 (99.0)	1.9 (98.8)	2.0 (99.0)
		MTBE	−0.85 (12.3)	1.6 (97.5)	2.2 (99.3)	1.6 (97.5)
MMQ	4.5	DEE	1.6 (97.5)	–	–	−1.4 (3.8)
		MDC	1.6 (97.5)	–	–	−2.9 (<1)
		MTBE	2.5 (99.7)	–	–	−1.2 (5.6)
MQ-IS		MTBE	–	1.4 (96.2)	1.3 (95.2)	0.84 (87.4)

*Phosphate buffer (0.3 M).

ammonium as counter-ion. The distribution ratio for MMQ at pH 9.00 is highly dependent on the chain length of the quaternary counter ion (Table III).

Choice of chromatographic support

There are wide differences in properties between the many brands of supports labelled as octadecyl silica (C_{18}). Several solid octadecyl supports (Table I) were tested for the separation of MQ, MMQ and MQ-IS, with the same mobile phase of acetonitrile–phosphate buffer (0.1 *M*, pH 2.50) (50:50, v/v). MQ shows good peak symmetry on all supports. Ultrasphere gives very short retention times for MQ and MQ-IS and very strong tailing for MMQ. The best support of those tested for the separation of MQ, MMQ and MQ-IS is Spherisorb ODS-1. Differences in retention time and selectivity using different supports and the same mobile phase (Table I) may result from differences in the silanol groups present in various reversed-phase supports and from differences in the pore size distribution. Recently Waters [9] and Walczak et al. [10] classified similar or equivalent supports on the basis of hydrophobicity and physical and chemical factors. By using these two reports the analyst can better define the supports used in published papers and select a similar or equivalent support for a given application.

Influence of the pH of the mobile phase

The pH of the mobile phase has a strong influence on the separation (Fig. 2). Since MMQ is a weak acid ($pK_a = 4.5$) it becomes highly retained when the pH is decreased because of a change in the degree of ionization of the carboxylic acid group. The selectivity for the separation of the metabolite and the parent drug is best at lower pH (pH less than 2.3 or between 3.3 and 4.0).

The stated pH of the mobile phase is the apparent pH measured by a combined glass electrode, calibrated with aqueous buffers of pH 4.0 and 7.0. Deviations occur between the measured pH of the mobile phase with high acetonitrile ratios and the actual pH, which is due to the change in the dissociation constants in water–organic solvent mixtures [11].

Influence of acetonitrile

The separation between MMQ and the other analytes improved with decreasing concentration of acetonitrile in the mobile phase, while the curves for MQ and MQ-IS were parallel (Fig. 3).

TABLE III

DISTRIBUTION RATIOS (LOGARITHMIC) OF MMQ EXTRACTED AS ION-PAIR WITH QUATERNARY AMMONIUM COMPOUNDS

Aqueous phase: phosphate buffer (0.3 *M*, pH 9.0)–quaternary ammonium ion (0.1 *M*). All data are means from one series with duplicate extraction experiments. Extraction recovery in parentheses.

Quaternary ammonium ion	Organic phase	Distribution ratio
Tetraethylammonium	DEE	−1.4 (<1)
Tetrabutylammonium	DEE	0.18 (60.2)
Tetrahexylammonium	DEE	2.0 (99.0)

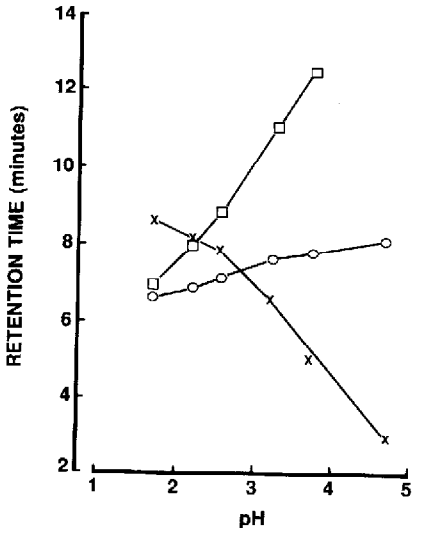

Fig. 2. Effect of pH of the mobile phase on retention time. Mobile phase, phosphate buffer (0.1 *M*)-acetonitrile (50:50, v/v); flow-rate, 1.0 ml/min; solid phase, Spherisorb ODS-1 (5 μm). Curves: ○ = MQ; × = MMQ; □ = MQ-IS.

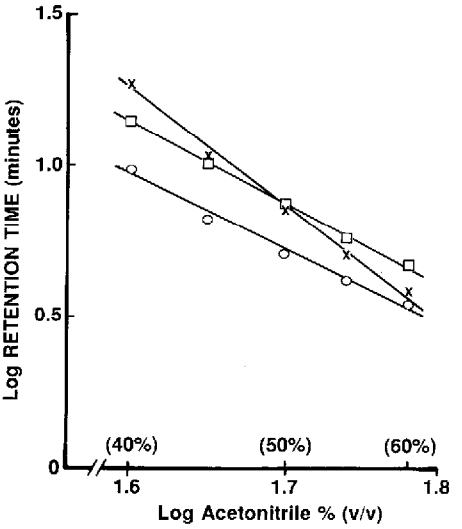

Fig. 3. Effect of acetonitrile content in the mobile phase on retention time in 0.1 *M* phosphate buffer (pH 2.50). Solid phase, Spherisorb ODS-1 (5 μm). Curves: ○ = MQ; × = MMQ; □ = MQ-IS.

Addition of amines

Amines of various sizes are often used as modifiers to suppress interaction of basic compounds with silanol groups. The addition of the cationic modifier DMOA to the mobile phase shortens the retention time for MQ, has no effect for MQ-IS and increases the retention time for MMQ (Fig. 4). Peak symmetry with Spherisorb ODS-1 is good (asymmetry factor 2) before adding the long-chain amine DMOA.

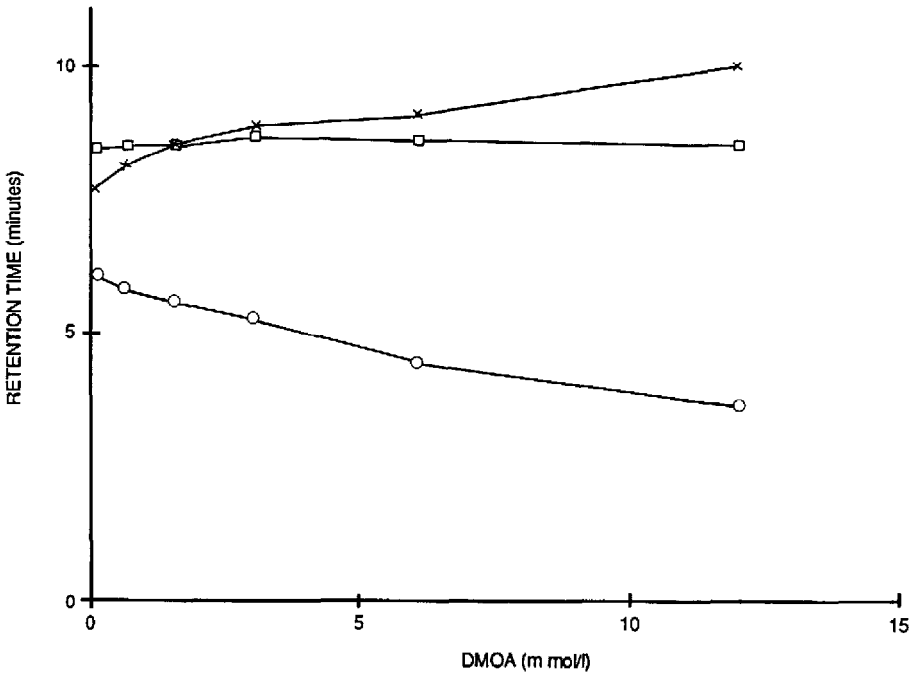

Fig. 4. Effect on retention time of DMOA. Mobile phase, phosphate buffer (0.1 *M*, pH 2.20)–acetonitrile (50:50, v/v); flow-rate, 1.0 ml/min; solid phase, Spherisorb ODS-1 (5 μm). Curves: ○ = MQ; × = MMQ; □ = MQ-IS.

DMOA gives a shorter retention time for MQ because it competes effectively with MQ for the adsorption sites on the support [12]. The retention time of MMQ increases because of an ion-pair distribution with DMOA to the support: since electroneutrality must be maintained on the support when DMOA is adsorbed, DMOA must be accompanied by ions of the opposite charge.

Optimizing separation conditions

The separation of MQ, MMQ and MQ-IS appears to be controlled by, among other factors, a combination of: (1) a pH effect, mainly due to the more highly retained protonated forms of MMQ; (2) interaction with silanol groups on the stationary phase, which can be largely eliminated by DMOA or some other amine in the mobile phase to avoid column-to-column changes in selectivity; (3) the percentage of acetonitrile in the mobile phase.

By using plots (Figs. 2–4) it was possible to select a mobile phase system for separation of MQ, MMQ and MQ-IS. In a typical chromatogram with Spherisorb ODS-1, all the compounds are well separated with baseline resolution (Fig. 5). The effect of varying the concentration of perchlorate (0–100 m*M*) in the mobile phase improved the separation somewhat. Retention times, however, were slightly increased for MQ, an effect typical of ion-pair separation.

Possible interferences of commonly used antimalarial drugs with the Spherisorb ODS-1 support, with acetonitrile–phosphate buffer (55:45, v/v, pH 2.20) were evaluated. None of the antimalarial drugs or metabolites listed in Table IV

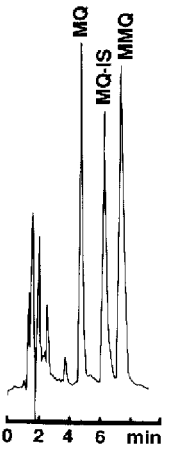

Fig. 5. Separation of MQ, MQ-IS and MMQ. Mobile phase, phosphate buffer (0.1 *M*, pH 2.20)–acetonitrile (55:45, v/v) with 3 m*M* DMOA; flow-rate, 1.0 ml/min; solid phase, Spherisorb ODS-1 (5 μm).

TABLE IV

RETENTION TIMES FOR SELECTED ANTIMALARIAL DRUGS AND METABOLITES

Mobile phase, phosphate buffer (0.1 *M*)–acetonitrile (45:55, v/v) pH 2.20; solid phase, Spherisorb ODS-1.

Antimalarial drug	Retention time (min)
MQ	8.01
MMQ	12.03
MQ-IS	9.62
Amodiaquine	3.15
Desethylamodiaquine	<3.0
Chloroquine	3.58
Desethylchloroquine	<3.0
Chlorcycloguanil	4.25
Chloroproguanil	6.93
Cycloguanil	3.04
Halofantrine	<3.0
Metoprine	4.47
Primaquine	<3.0
Proguanil	4.89
Pyrimethamine	4.34
Quinine	<3.0
Sulfadoxine	3.73

was found to elute sufficiently close to MQ, MMQ or the MQ-IS to cause interference. This chromatographic system can, therefore, be used for simultaneous determination of MQ and MMQ when patients are treated with other antimalarial drugs in combination with MQ.

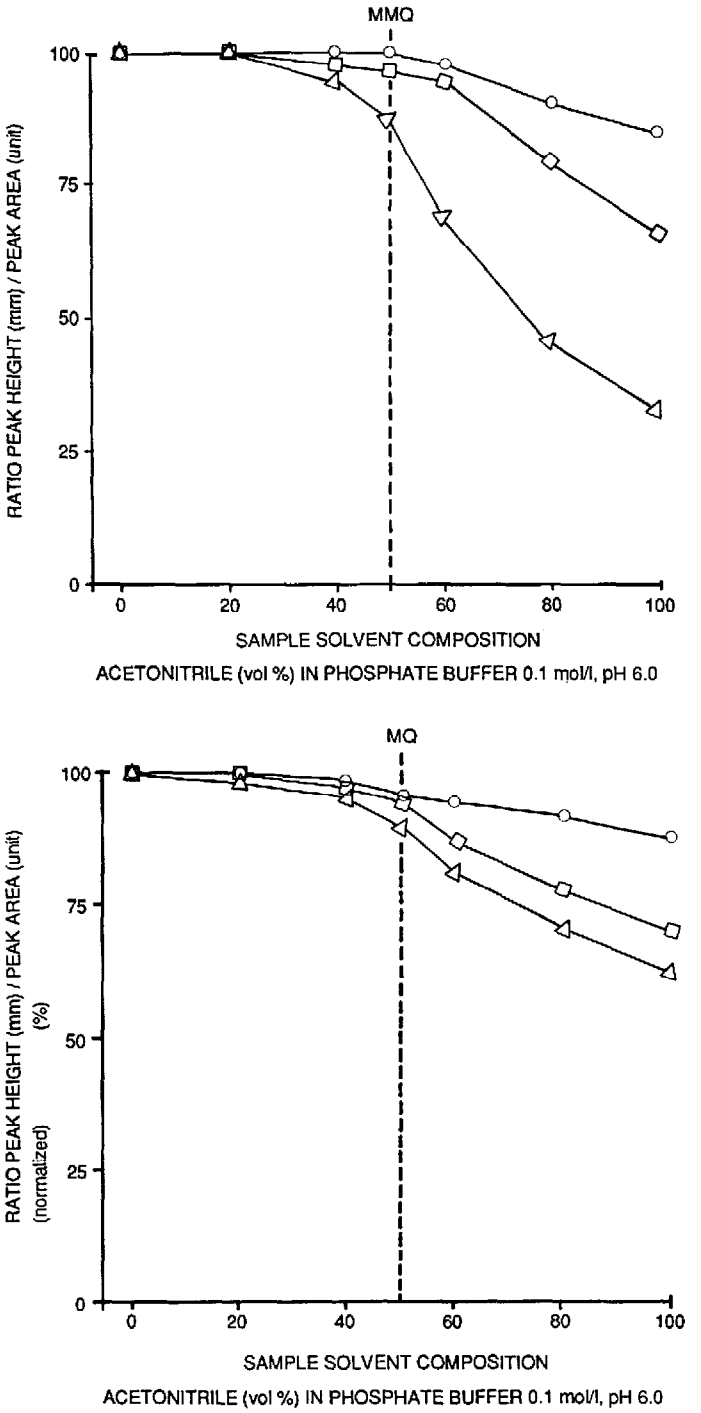

Fig. 6. Effect of acetonitrile–phosphate buffer (0.1 *M*, pH 6.0) as analyte solvent on the ratio of peak height to peak area and varying injection volume. The ratio is normalized to a value of 100% at a sample solvent composition of 0% acetonitrile. The dotted line corresponds to the mobile phase composition. Mobile phase, phosphate buffer (0.1 *M*, pH 2.50)–acetonitrile (50:50, v/v); flow-rate, 1.0 ml/min; solid phase, Spherisorb ODS-1 (5 μm). Injection volume: △ = 100 μl; □ = 50 μl; ○ = 25 μl.

Solvent for the residue

In HPLC, problems arise concerning the nature of the solvent used for reconstituting the sample after evaporation, involving different peak responses, distortions or peak splitting [13,14]. For both MQ and MMQ, the peak height/peak area ratio decreased as the concentration of acetonitrile used for reconstituting the sample was increased (Fig. 6). The peak-area variation was ca. 5% over the range 0–100% acetonitrile in phosphate buffer (0.1 *M*, pH 6.0) as sample solvent for both MQ and MMQ. However, over the same range the peak-height decrease was more than 40% for MQ and 50% for MMQ at 50-μl injection volume. The molar absorptivity at the wavelength (285 nm) for MQ in phosphate buffer (0.1 *M*, pH 6.0) as a function of acetonitrile concentration changes in the same manner as peak area. The amounts injected were in the 0.07–0.32 μg range to minimize column saturation effects and remain within the linear range for the detector. The coefficient of variation of retention time during the experiment for both MQ and MMQ was below 0.45%, as the injection solvent composition was varied from 0 to 100% acetonitrile.

It is clear that the solvent can affect the chromatographic evaluation of MQ and MMQ, especially when peak heights are used for calculation. Standard and sample solvent solutions must be closely matched in composition, and the solvent composition used must optimize the peak heights for MQ and MMQ.

HPLC injection solvents temporarily take part in the liquid–liquid equilibrium or the liquid–solid equilibrium in the column. Ng and Ng [13] described these solvent effects by computer simulation using an elution model in which a segment of solvent has a different elution strength from that of the mobile phase. This model explains some kinds of gradient elution effects.

In going from 100% water to 100% acetonitrile, the sample spreads out more in the top of the column. This results in decreased peak heights as the amount of acetonitrile increases. The effect is similar to injecting a larger volume.

Work is in progress to apply the chromatographic and extraction systems described in this paper to the simultaenous determination of MQ and MMQ in plasma and whole blood by liquid chromatography, and to the determination of MQ from capillary blood collections on filter paper by gas–liquid chromatography with electron-capture detection.

ACKNOWLEDGEMENTS

We thank the Sweden–American Foundation for a grant to Yngve Bergqvist in support of his tenure as a Guest Researcher at the Centers for Disease Control, Roche-Produkter (Sweden) for the gift of MQ, MMQ and MQ-IS, and Dr. Anders Sokolowski for his comments on the manuscript.

REFERENCES

1 D.E. Schwartz and V.B. Ranalder, Biomed. Mass Spectrom., 8 (1981) 589–592.
2 R. Heizmann and R. Geschke, J. Chromatogr., 311 (1984) 411–417.
3 D. Dadgar, J. Climax, R. Lambe and A. Darragh, J. Chromatogr., 337 (1985) 47–54.

4 J.M. Grindel, P.F. Tilton and R.D. Shaffer, J. Pharm. Sci., 66 (1977) 834–836.
5 I.M. Kapetanovic, J.D. Digiovanni, J. Bartosevich, V. Melendez, J. von Bredow and M. Heiffer, J. Chromatogr., 277 (1983) 209–215.
6 P.J. Arnold and O.V. Stetten, J. Chromatogr., 353 (1986) 193–200.
7 D.E. Schwartz, in A. Frigerio and M. McCamish (Editors), Recent Developments in Chromatography and Electrophoresis, Elsevier, Amsterdam, 1980, pp. 69–72.
8 G. Schill, H. Ehrsson, J. Vessman and D. Westerlund, Separation Methods, Swedish Pharmaceutical Press, Stockholm, 1983.
9 M.J. Waters, J. Assoc. Off. Anal. Chem., 70 (1987) 465–469.
10 B. Walczak, L. Morin-Allory, M. Lafosse, M. Dreux and J.R. Chretien. J. Chromatogr., 395 (1987) 183–202.
11 B.A. Bidlingmeyer, J. Chromatogr. Sci., 18 (1980) 525–539.
12 A. Sokolowski and K.G. Wahlund, J. Chromatogr., 189 (1980) 299–316.
13 T.L. Ng and S. Ng, J. Chromatogr., 329 (1985) 13–24.
14 K.C. Chan and E.S. Yeung, J. Chromatogr., 391 (1987) 465–467.