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SIMULTANEOUS DETERMINATION OF CHLOROQUINE AND ITS DESETHYL METABOLITE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY

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SUMMARY

A gas chromatographic method for the simultaneous determination of chloroquine and its metabolite desethylchloroquine in plasma is described. Chloroquine, desethylchloroquine and internal standard are extracted as bases with *n*-hexane—pentanol (90:10) and then back-extracted to an acid aqueous phase. The aqueous phase is made alkaline and after re-extraction into chloroform and evaporation of the chloroform, acylation with trifluoroacetic anhydride is performed. Separation is achieved on an OV-17 column at 250°C. Chloroquine and desethylchloroquine can be determined down to 0.1—0.2 μmol/l (30—60 ng/ml), with a coefficient of variation of 12%, using a nitrogen detector. The method shows good correlation (r = 0.98) with a recently developed liquid chromatographic method.

INTRODUCTION

Chloroquine (CQ) is one of the most frequently used antimalarial agents and is furthermore used as an effective drug in the treatment of rheumatoid arthritis. Desethylchloroquine (CQM) has been identified in urine as the main metabolite of CQ using thin-layer chromatography [1, 2] and by gas chromatography—mass spectrometry [3]. A relationship between serum levels of CQ and the frequency of side-effects has been shown in the treatment of rheumatoid arthritis with CQ [4]. Side-effects may be seen for a plasma concentration of CQ exceeding about 0.8—1.0 μmol/l. A number of different analytical methods for the determination of CQ have been proposed. These include fluorimetry [5], gas—liquid chromatography [6] and spectrodensitometry [7]. These methods do not adequately quantify CQ and CQM separately. A sensitive assay for the determination of plasma levels of CQ and CQM by high-performance liquid chromatography (HPLC) has recently

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been presented [8], which made it possible to study the bioavailability of CQ and its pharmacokinetics in man [9].

In this paper, a selective gas chromatographic method for the simultaneous determination of CQ and CQM using a nitrogen detector is described. It can be used for routine determinations of CQ and CQM levels in patients undergoing chronic treatment with chloroquine as an alternative to the perhaps more demanding HPLC method. The method is documented with respect to precision, recovery at different plasma levels, and clinical cross-testing with a liquid chromatographic method.

MATERIALS AND METHODS

Chemicals and reagents

Chloroquine, desethylchloroquine and 7-iodo-4-(1-methyl-4-diethylaminobutylamino)quinoline, used as the internal standard (IS), were kindly supplied by Sterling-Winthrop, Skärholmen, Sweden. The molecular structures are shown in Fig. 1. *n*-Hexane, 1-pentanol and chloroform were of analytical grade from Merck (Darmstadt, G.F.R.). Trifluoroacetic anhydride (TFA) purum was from Fluka (Buchs, Switzerland). All other chemicals were of analytical or equivalent grade and were used without further purification. The glass utensils used were cleaned with nitric acid (2 mol/l) in an ultrasonic bath, followed by a rinse with deionized, Milli Q filtered water (Millipore, Bedford, MA, U.S.A.).

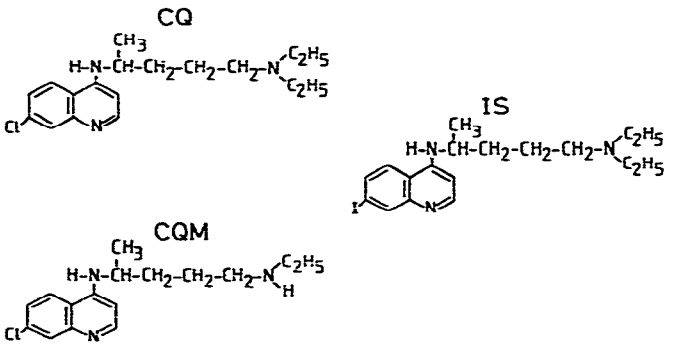

Fig. 1. Chemical structures of chloroquine (CQ), desethylchloroquine (CQM) and internal standard (IS).

Gas chromatography

The analyses were performed on a Perkin-Elmer 990 gas chromatograph connected to a Perkin-Elmer nitrogen—phosphorus detector. The glass column (2 m × 2 mm I.D.) was silanized and packed with 3% OV-17 on Gas-Chrom Q, 80—100 mesh, from Applied Science Labs. (State College, PA, U.S.A.). Nitrogen, purified with a molecular sieve, was used as carrier gas at a flow-rate of 40 ml/min and the column temperature was 250°C. The injector and detector were both held at 300°C. The flow-rates of hydrogen and air to the detector were 5 ml/min and 70 ml/min, respectively.

Mass spectrometry

A Varian MAT 44 S mass spectrometer was used, coupled to a Varian 3700 gas chromatograph equipped with a glass column filled with the same packing as above. The ionization energy was 70 eV.

Extraction and derivatisation procedure

A 2.0-ml aliquot of the plasma sample, or urine diluted 1:20, and 0.100 ml of the internal standard solution (50 μmol/l) were made alkaline with 1.0 ml of sodium hydroxide (1 mol/l) and extracted for 15 min with 5.0 ml of *n*-hexane—1-pentanol (90:10). After centrifugation for 10 min, 4.0 ml of the organic phase were transferred to another tube and 5.0 ml of hydrochloric acid (0.2 mol/l) were added. The tube was shaken for 15 min and centrifuged. Then 4.0 ml of the aqueous phase were made alkaline by addition of 0.5 ml of sodium hydroxide (5 mol/l), and extracted with 5.0 ml of chloroform for 15 min. After centrifugation 4.0 ml of the chloroform phase were evaporated to dryness. The residue was dissolved in 50 μl of ethyl acetate and 10 μl of TFA were added. The tube was incubated for 15 min at 50°C, followed by evaporation to dryness. The residue was dissolved in 25 μl of ethyl acetate, and 2 μl were injected into the gas chromatograph.

Calibration curves were prepared each day by analysis of samples with known amounts of CQ and CQM added to blank plasma. Curves in the concentration range of 0.2—2 μmol/l are shown in Fig. 2.

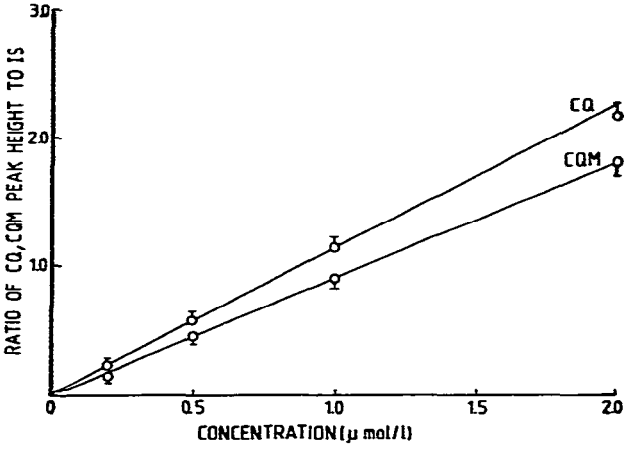

Fig. 2. Calibration curves of chloroquine (CQ) and desethylchloroquine (CQM) obtained after extraction from plasma. Each point represents the mean value of three replicate determinations, and the length of the vertical bars denotes the corresponding standard deviation.

Precision

The precision of the method was determined by analysing pooled plasma.

Extraction efficiency

CQ and CQM were added in the concentration range 0.2—1.0 μmol/l to a pool of plasma from patients undergoing chloroquine therapy or to a phos-

phate buffer (pH 7.0) containing 154 mmol/l sodium chloride. The samples were then analyzed by the present method.

Selectivity

The selectivity of the gas chromatographic determination of CQ and CQM was verified by comparison with an ion-pair liquid chromatographic method with fluorescence detection [8].

RESULTS AND DISCUSSION

Extraction conditions

Distribution conditions for CQ, CQM and internal standard in buffered aqueous solutions using *n*-hexane—1-pentanol (90:10) or chloroform as organic phase have been studied [10]. Quantitative extraction as bases (99%) for equal phase volumes is obtained when the pH of the aqueous phase is >10. For practical reasons, a mixture of *n*-hexane—1-pentanol was chosen in the first extraction step since the organic phase will then be the upper phase and transfer to a new test tube is thus facilitated. The back-extraction to an acid aqueous phase is quantitative for pH <5. Repeated extraction is necessary to obtain a sufficiently pure extract for subsequent work.

Derivatisation conditions

Several stationary phases (OV-7, OV-17, QF-1 and OV-210) were tried in order to separate the tertiary amine CQ from CQM, which is a secondary amine. However, none of these phases adequately separated CQ and CQM. By performing a TFA derivatisation, the retention time is changed for CQM but not for CQ and the internal standard, as is illustrated in Fig. 3. This figure

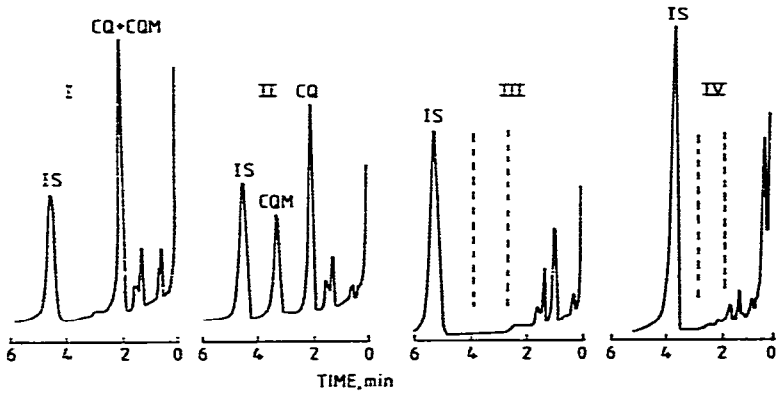

Fig. 3. Gas chromatograms of plasma samples containing chloroquine (CQ), desethylchloroquine (CQM) and internal standard (IS). (I) Plasma sample without TFA derivatisation containing CQ 0.44 μmol/l, CQM 0.37 μmol/l and internal standard. (II) The same plasma sample as in I after TFA derivatisation. (III) Plasma blank with internal standard. Retention times for CQ and CQM at dotted lines. (IV) Urine blank with internal standard. Retention times for CQ and CQM at dotted lines.

also shows that neither plasma nor urine contain endogenous substances that interfere.

The mass spectrum of TFA-CQM is presented in Fig. 4. The molecular ion (*m/z* 387) and the fragmentation pattern indicate that only one trifluoroacetyl group is introduced into the metabolite. The fact that the chromatographic behaviour of CQ and IS is unaffected by the derivatisation procedure indicates that it is the nitrogen on the side-chain which reacts with TFA. This is further corroborated by the mass spectrum of TFA-treated CQ which contained no peak with *m/z* exceeding 319.

The formation of the TFA-CQM derivative is complete within 15 min at 50°C and the product is stable under the reaction conditions for at least 4 h. After evaporation of the excess reagent, the derivative was found to be stable for more than one week at room temperature.

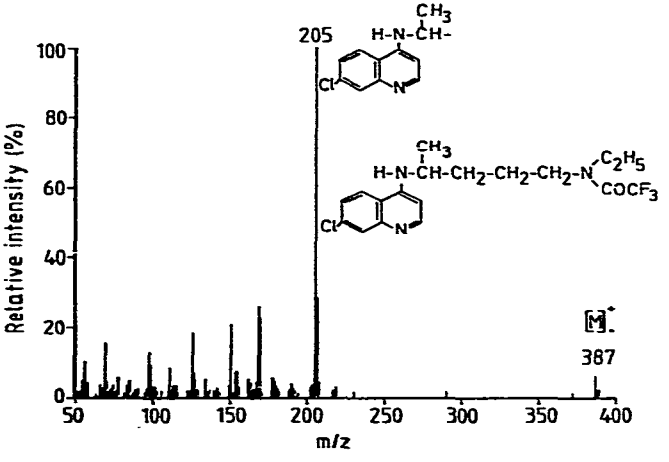

Fig. 4. Mass spectrum of trifluoroacetylated desethylchloroquine. Ions below *m/z* 50 are omitted. Ionization energy = 70 eV.

Sensitivity, precision and recovery

It was possible to determine 0.2 μmol/l CQ and CQM with a relative standard deviation of 12%. Results are shown in Table I. Repeated injections of

TABLE I

PRECISION OF THE METHOD USING POOLED PLASMA

	Concentration (μmol/l)	R.S.D.* (%)	*n*
Chloroquine	0.79	5.8	7
	0.22	11.6	7
Desethylchloroquine	0.77	6.5	7
	0.22	12.3	7

*R.S.D. = relative standard deviation.

the same extract of CQ and TFA-CQM gave a coefficient of variation of 2.1% (n = 10) for both CQ and CQM at the 0.35 μmol/l level. The recovery of CQ and TFA-CQM was about 50% at the 2 μmol/l level. It was estimated from a comparison between the peak heights of extracted plasma standards and those from known amounts of the compounds injected directly into the gas chromatograph. The observed recovery is close to the value (51%) calculated from the extraction procedure. The recoveries of CQ and CQM from spiked plasma samples and an aqueous medium are equal. As shown in Fig. 5, the standard addition plots have the same slopes for both matrices.

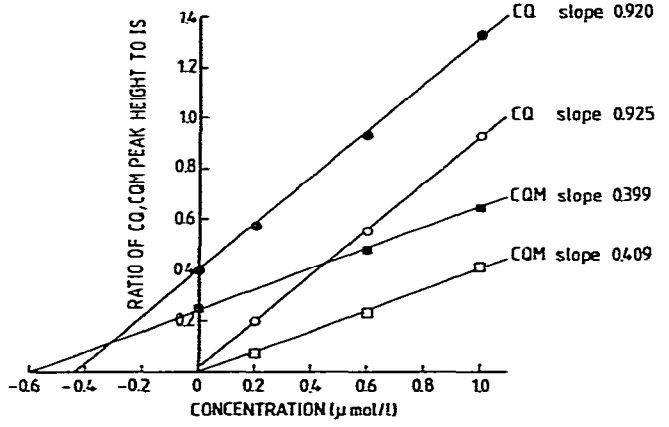

Fig. 5. Comparison of the recovery of chloroquine (CQ) and desethylchloroquine (CQM) from plasma and aqueous solution. Each point represents the average of two determinations. (•), CQ added to a plasma pool from patients; (○), CQ added to a phosphate buffer (pH 7.0) containing 154 mmol/l sodium chloride; (▪), CQM added to a plasma pool from patients; (□), CQM added to a phosphate buffer (pH 7.0) containing 154 mmol/l sodium chloride.

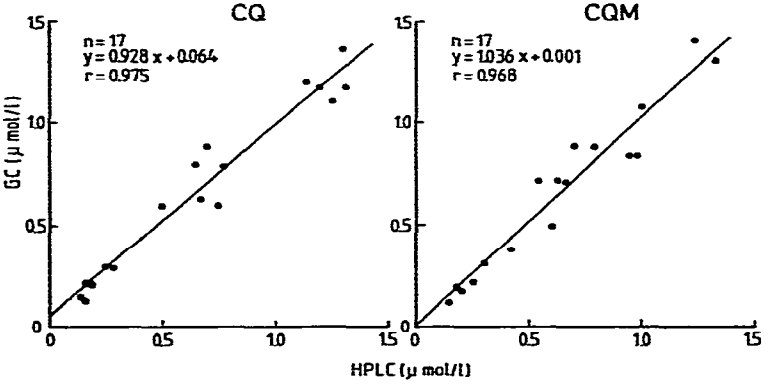

Fig. 6. Comparison of results obtained by liquid chromatography (x) and gas chromatography (y) for chloroquine (CQ) and desethylchloroquine (CQM) in plasma. Samples were taken from patients undergoing chloroquine therapy.

Stability of plasma samples

The influence of storage time on the CQ and CQM levels were determined from ten plasma samples stored for about 11 months at −20°C. No degradation of the compounds was observed within the precision of the gas chromatographic method (± 5% of the actual plasma levels).

Selectivity of the present method

The results of the selectivity are shown in Fig. 6. Since the HPLC and gas chromatographic methods use different methods for extraction, chromatography and detection, the high correlation coefficient demonstrates the good selectivity of the present method.

We have shown experimentally that the following frequently used drugs for treatment of rheumatic diseases — phenylbutazone, naproxen, prednisolone, salicylazosulphapyridine, ibuprofen, indomethacin and salicyclic acid — do not interfere with the present method.

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