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GAS CHROMATOGRAPHIC ANALYSIS OF CHLOROQUINE AFTER A UNIQUE REACTION WITH CHLOROFORMATES

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SUMMARY

A specific method for the gas chromatographic determination of chloroquine (CQ) after derivatization with chloroformates, using 9-bromophenanthrene as the internal standard and a column filled with 3% OV-17 on 80—100 mesh Supelcoport is described. Derivatization with chloroformates produced a pyrrolidine derivative, 4-(2-methyl-1-pyrrolidyl)-7-chloroquinoline with CQ, and a carbamate with desethylchloroquine. The chloroformate reaction for CQ is thus selective in the presence of CQ metabolites. The method based on flame ionization detection is highly suitable for quantitation of CQ in urine.

INTRODUCTION

Chloroquine (CQ) has a worldwide use in the therapy of malaria [1], is a potent antirheumatic agent [2] and is effective in the treatment of lupus erythematosus [3]. The analysis of CQ, a secondary aromatic amine with a tertiary aliphatic amine side-chain, in biological fluids and tissues has been performed mainly by a fluorometric method [4, 5], which requires prior removal of metabolites that otherwise would be determined as CQ. Recently, a liquid chromatographic method was described for CQ and its metabolite desethylchloroquine [6]. Gas chromatographic (GC) methods previously described for CQ [7, 8] did not take into consideration the potential interference from this metabolite which, as demonstrated in the present study, elutes together with CQ. Also, these methods do not have the sensitivity

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required for analysis in blood. An improved GC method was therefore sought, based on the well known reaction of chloroformates with tertiary amines, yielding carbamates [9—11].

This paper describes the unique reaction of CQ with chloroformates to form a pyrrolidine derivative, 4-(2-methyl-1-pyrrolidyl)-7-chloroquinoline, and the carbamate of diethylamine (Fig. 1).

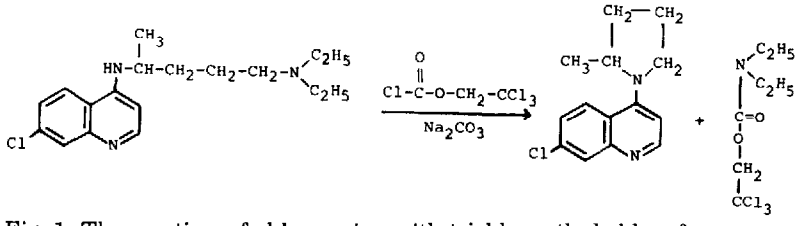

Fig. 1. The reaction of chloroquine with trichloroethyl chloroformate.

EXPERIMENTAL

Standards and reagents

Chloroquine phosphate and desethylchloroquine were kindly supplied by Sterling-Winthrop Research Labs. (Rensselaer, NY, U.S.A.). Trichloroethyl, methyl, ethyl, benzyl and isobutyl chloroformates and 9-bromophenanthrene were obtained from Aldrich (Milwaukee, WI, U.S.A.). Sodium carbonate, anhydrous, was obtained from Baker (Phillipsburg, NJ, U.S.A.). Nanograde methylene chloride was obtained from Fisher (Silver Springs, MD, U.S.A.). The alcoholic alkali solution was 0.5 *M* potassium hydroxide in methanol. Aqueous reagents were prepared from glass-distilled water and stored in glass bottles with PTFE-lined caps.

Syntheses

4-(2-Methyl-1-pyrrolidyl)-7-chloroquinoline was synthesized by a modification of a previously described method [12]. The side-chain precursor, 4-amino-1-pentanol, was prepared by reduction of 1-hydroxy-4-pentanone oxime with sodium and absolute ethanol. 4,7-Dichloroquinoline was then combined with the 4-amino-1-pentanol and heated neat, to yield 4-(4-hydroxy-1-methylbutylamino)-7-chloroquinoline, which upon action of hydrobromic acid and heat gave the pyrrolidine compound. The final product as well as the intermediates was characterized by melting point and mass spectrometry (MS).

Trichloroethyl N,N-diethylcarbamate was synthesized by dropwise addition of excess diethylamine in tetrahydrofuran to trichloroethyl chloroformate, also in tetrahydrofuran. Excess diethylamine and tetrahydrofuran were removed by evaporation. Identity was confirmed by mass spectral analysis.

Instruments

GC. A Varian Model 1440 gas chromatograph equipped with a flame ionization detector was used. The column (180 cm × 2 mm I.D.) was made of pyrex glass and packed with 3% OV-17 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min. Air and hydrogen flow-rates for the flame ionization detector

were 300 and 40 ml/min, respectively. The column temperature was 230°C (110°C for identification of carbamates of diethylamine) and the injector and detector temperatures were 240°C and 265°C, respectively.

GC—MS. Mass spectra were obtained with an LKB 9000S gas chromatograph—mass spectrometer operated at an accelerating voltage of 3.5 kV, ionization voltage of 20 eV, and a trap current of 60 μA. The column was the same as above. Fragment ions with a relative intensity of less than 5% were omitted for simplicity.

Examination of derivatization conditions

Reaction conditions were studied using the following derivatization procedure. CQ base was prepared from an alkalinized solution of CQ phosphate in water extracted with methylene chloride. The organic phase was evaporated to dryness under a gentle stream of nitrogen. To 200 μg of CQ base and 25 μg of 9-bromophenanthrene as internal standard in 200 μl of methylene chloride were added 10 μl of isobutyl chloroformate and about 10 mg of anhydrous sodium carbonate. The mixture was left at room temperature; 1—2 μl of the organic phase were injected at intervals into the gas chromatograph. The peak height ratio of the formed derivative to internal standard was measured. The percentage yield of derivative was calculated relative to maximum yield. The influence of solvent, temperature and the amounts of isobutyl chloroformate and sodium carbonate on the reaction rate was investigated, as were the comparative reactivities of methyl, ethyl, benzyl and trichloroethyl chloroformates.

Prior to GC the reaction products from CQ and isobutyl chloroformate were examined by thin-layer chromatography [silica gel GF; solvent system 25% ammonia—methanol (3:200), UV detection] and compared to CQ and synthetic 4-(2-methyl-1-pyrrolidyl)-7-chloroquinoline.

The reaction of desethylchloroquine with isobutyl chloroformate and its separation from CQ by GC were also examined.

Standard curves

Varying quantities of CQ (10—400 μg) and 10 or 50 μg of 9-bromophenanthrene as internal standard were added to 1.0-ml samples of human urine in 15-ml round-bottomed centrifuge tubes. The urine was made alkaline (pH > 13) with an equal volume of 1 *N* sodium hydroxide in 10% sodium chloride, and extracted on a reciprocating shaker for 10 min with 10 ml of methylene chloride. After centrifuging for 5 min at 2000 *g*, the organic phase was transferred to a conical centrifuge tube and evaporated to dryness in a water bath at 35°C under a gentle stream of nitrogen. The residue was reconstituted in 200 μl of methylene chloride; about 10 mg anhydrous sodium carbonate and 10 μl of isobutyl chloroformate were added and the mixture was left at room temperature for 1 h with the tubes tightly capped. Excess chloroformate was hydrolyzed by shaking the reaction mixture vigorously for 30 sec with 0.5 ml of 0.5 *M* alcoholic alkali solution. After the addition of 0.5 ml of water, shaking and centrifugation, the aqueous phase was discarded. A 1—2 μl aliquot of the organic phase was injected into the gas chromatograph.

RESULTS AND DISCUSSION

The reaction of CQ with trichloroethyl chloroformate resulted in a product with a GC retention time considerably shorter than that for the underivatized drug, relative retention 0.33 (Fig. 2B compared to Fig. 2A). The shorter retention time suggested a significant reduction of the molecular weight of CQ. The electron impact mass spectrum of this peak suggested it to be 4-(2-methyl-1-pyrrolidyl)-7-chloroquinoline (Fig. 3). The retention time and mass spectrum of the synthetic reference compound confirmed this observation. An additional GC peak associated with CQ was observed when the column temperature was decreased from 230°C to 110°C. The mass spectrum of this peak compared to that of a synthetic standard demonstrated it to be trichloroethyl N,N-diethylcarbamate (Fig. 4).

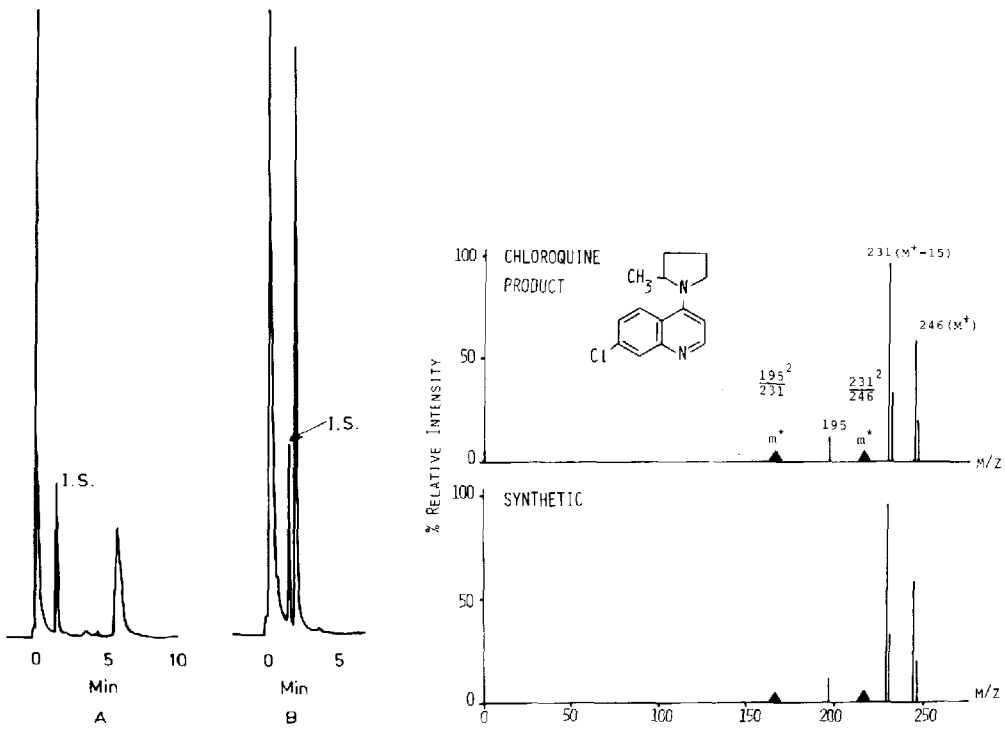

Fig. 2. Gas chromatograms of chloroquine (200 μg/ml) (A) before and (B) after chloroformate reaction with 9-bromophenanthrene (25 μg/ml) as internal standard (I.S.). Column temperature 230°C.

Fig. 3. Mass spectra of one of the derivatization products of chloroquine and synthetic 4-(2-methyl-1-pyrrolidyl)-7-chloroquinoline. An m* indicates intense metastable ions.

It is postulated that the initial reaction between CQ and trichloroethyl chloroformate yields the reactive intermediate quaternary ammonium ion generally expected from tertiary aliphatic amines and chloroformates [11]. This intermediate ion may then directly cyclize to the pyrrolidine derivative with the loss of trichloroethyl N,N-diethylcarbamate (Fig. 1). Deamination

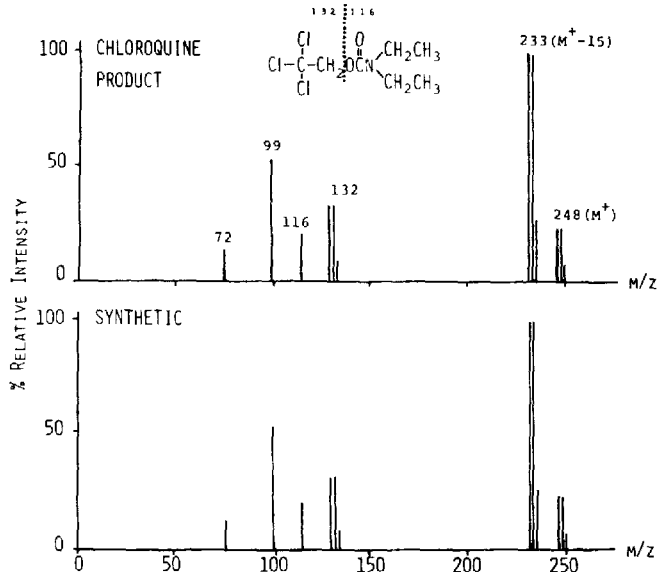

Fig. 4. Mass spectra of one of the derivatization products of chloroquine and synthetic trichloroethyl N,N-diethylcarbamate.

of the intermediate ion under formation of an organic chloride, 4-(4-chloro-1-methylbutylamino)-7-chloroquinoline in a similar fashion as shown for other tertiary amines [9, 11, 13, 14] is, however, another possibility. Such a chloride may then react with the secondary aromatic amino group, e.g. in the hot injector of the gas chromatograph, resulting in the observed cyclization. However, this chloride has been shown in the synthesis of CQ analogues [12] to be quite stable as opposed to the corresponding bromide. Moreover, it was suggested, although not unequivocally proven, by thin-layer chromatography that the pyrrolidine derivative was formed prior to the GC separation, i.e. the R_F value of the reaction product was identical to that of synthetic 4-(2-methyl-1-pyrrolidyl)-7-chloroquinoline.

When reacting CQ with several other chloroformates, e.g., methyl, ethyl, isobutyl and benzyl chloroformates, the identical cyclized pyrrolidine product was formed in addition to the corresponding carbamates of diethylamine as evidenced by GC—MS. Thus, the cyclization of CQ is a common reaction induced by all chloroformates.

The isobutyl chloroformate was the fastest reacting chloroformate, leading to complete reaction within 45 min, while the reaction with the methyl or ethyl chloroformates was complete within 2 h and after 12 h with benzyl or trichloroethyl chloroformates, when performed at room temperature using methylene chloride as the solvent and sodium carbonate as the base catalyst. In the absence of sodium carbonate the yield of the reaction with isobutyl chloroformate after 45 min was only 14%. Whereas the reaction rate in ethylene chloride and methylene chloride was about the same, it was significantly reduced in less polar solvents, e.g., hexane, heptane and benzene. Raising the reaction temperature to 50°C had no apparent effect. The pyrrolidine derivative of CQ was stable for at least 48 h.

The reaction between isobutyl chloroformate and CQ leading to the pyrrolidine derivative was highly reproducible and appeared quantitative based on comparisons with the synthetic pyrrolidine derivative. One-ml urine samples containing between 10 and 400 μg of CQ were extracted and derivatized as described in the Experimental section. There were no interferences from the biological material. The standard curve obtained was linear and passed through the origin (correlation coefficient 0.998). The coefficients of variation at the 10 and 400 μg/ml levels were 9.6% and 13%, respectively. These data indicate that this method, based on flame ionization detection, is highly suitable for quantitation of CQ in urine. It has been suggested that about one third of the administered dose of CQ is excreted unchanged in urine [15].

One of the potential problems associated with the determination of CQ in both urine and blood is the interference by CQ metabolites in the assay procedure. Without derivatization the main CQ metabolite, desethylchloroquine [15], did not separate from CQ by GC using either OV-17 or OV-1 columns. Derivatization with isobutyl chloroformate, however, produced the pyrrolidine derivative with CQ and a carbamate with desethylchloroquine, the latter derivative with a much longer retention time (t_{rel} = 3.2 compared to underivatized CQ and 9.7 compared to the pyrrolidine derivative of CQ). Structure confirmation was carried out by GC—MS. The chloroformate reaction for CQ is thus highly selective in the presence of CQ metabolites.

The chloroformate reaction with CQ appears to have several other advantages only partially explored. The cyclization of CQ markedly reduced problems of adsorption to the GC system, probably because of elimination of the highly basic aliphatic tertiary amino group. The increased volatility of the pyrrolidine derivative permitted lower column temperatures to be used for its elution. Both of these factors should promote the GC of very small sample sizes of CQ. The sensitivity of detection of the pyrrolidine derivative can be greatly enhanced, in particular by MS detection. Preliminary results also indicate that the yield of the trichloroethyl N,N-diethylcarbamate from the reaction of trichloroethyl chloroformate with CQ is also quantitative and this derivative is detectable in nanogram quantities by electron-capture detection.

To test whether the chloroformate reaction described for CQ is also applicable to other antimalarial drugs with an identical side-chain, derivatization of quinacrine was examined. Exactly the same cyclization as with CQ was found.

More generally, the results of this study add to the experience with chloroformates as derivatizing reagents for tertiary amines. Although the mechanism for the formation of the initial intermediate reaction products may be the same for all tertiary amines, the end products may differ substantially dependent on the structure of the individual molecules [9—11, 13, 14, 16] as further illustrated by our findings with CQ.

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