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ELECTRON-CAPTURE GAS CHROMATOGRAPHIC ASSAY FOR PRIMAQUINE IN BLOOD*

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SUMMARY

A sensitive gas chromatographic method for the quantitative determination of the anti-malarial drug primaquine is described. The method involves derivatization with heptafluorobutyric anhydride to form the diheptafluorobutyramide derivative after a single extraction at alkaline pH. The derivatives are quantitated by electron-capture gas chromatography. Blood levels of primaquine as low as 8 ng/ml can be measured with good precision.

INTRODUCTION

The antimalarial drug primaquine, 8-(4-amino-1-methyl-butylamino)-6-methoxyquinoline is the drug of choice in the treatment of *Plasmodium vivax* malaria and is being increasingly used in the chemotherapy of chloroquine-resistant forms of malaria [1]. The pharmacokinetic profile of primaquine in animals and man is largely unknown due to the lack of sensitive and specific assay methods for the determination of primaquine in biological fluids. Recently Greaves et al. [2] have described a method for the analysis of primaquine in plasma and urine using gas chromatography—mass spectrometry (GC—MS). The lower limit for quantitation by this method is 50 ng/ml of plasma or urine and measurement of intraerythrocytic primaquine levels has not been successful with this method. This paper describes an analytical method for the measurement of primaquine at a concentration of 10 ng/ml and above in blood or plasma by selective extraction, derivatization using heptafluorobutyric (HFB) anhydride to form the diheptafluorobutyramide (diHFB) derivative and subsequent separation and quantitation using GC with electron-capture detection.

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EXPERIMENTAL

Solvents, standards and reagents

Reagent grade cyclohexane and benzene were obtained from Glaxo Laboratories (India) Ltd., Bombay, India; methylene chloride and methanol Uvasol® were from E. Merck, Bombay, India.

Cyclohexane and benzene were purified by washing with concentrated sulphuric acid until free from colour, followed by successive washings with water, dilute sodium hydroxide and water. The washed solvents were dried over calcium chloride and distilled using a 120-cm Vigreux column before use. Methylene chloride was also distilled using the Vigreux column and stored in the refrigerator. Heptafluorobutyryl chloride and heptafluorobutyric anhydride were obtained from Fluka (Buchs, Switzerland). Primaquine diphosphate was from Aldrich (Milwaukee, WI, U.S.A.) and the internal standard diphosphate was synthesised according to the previously reported procedure [3]. The column packing material, 3% SP-2401 on 80—100 mesh Supelcoport, was from Supelco (Bellefonte, PA, U.S.A.). Ultra high purity nitrogen (IOLAR-2) used as carrier gas was from Indian Oxygen Ltd. (Bombay, India).

All other chemicals used were of analytical reagent grade obtained locally.

Apparatus

The gas chromatograph was a Pye-Unicam Model 204 equipped with a 10 mCi ^{63}Ni electron-capture detector. Nitrogen was used as the carrier gas at a flow-rate of 46 ml/min. The column was operated at 235°C, the injector at 200°C and the detector at 300°C. Peak areas were measured by the height × width at half-height method. The samples were injected "on column" using the solvent-flush technique.

All the glassware used in processing the samples and the glass column were silanized to prevent adsorption by soaking overnight in a 2.5% solution of dimethyldichlorosilane in toluene followed by two rinses with distilled toluene and a final rinse with distilled methanol. They were dried at 90—100°C before use. Solvents and glassware used in the method were checked at random for contamination by carrying out blank runs through the entire method.

The glass column (2.1 m × 4 mm I.D.) was packed with 3% SP-2401 on 80—100 mesh Supelcoport. The packed column was conditioned without being connected to the detector for two to three days at 270°C using a carrier flow-rate of 40 ml/min. After this four to six injections of 10 μl of Silyl 8 (Pierce, Rockford, IL, U.S.A.) were given at 30-min intervals at a column temperature of 250°C. The conditioned column was kept at this temperature overnight and was primed with a mixture of primaquine diHFB and internal standard diHFB at a concentration of 1—2 ng/μl. After 15—20 priming injections, the column was connected to the detector and the response to injections of 0.4 ng of standard diHFB derivatives of primaquine and internal standard was checked. If the response was poor, priming and conditioning injections were repeated until the optimal response was obtained. In our experience it takes about eight to ten days to prepare a column that exhibits optimal sensitivity. After repeated use the column material may require topping-up, and this was easily accomplished by removing the top 2—3 cm of the packing and replacing it with fresh packing material.

Synthesis of mono- and diheptafluorobutyramide derivatives of primaquine and internal standard

The melting points reported here are uncorrected. Infrared (IR), ultraviolet (UV) and mass spectra were recorded, respectively, on a Perkin-Elmer Infracord, a Beckmann DK-2A and a Varian MAT CH-7. The general procedures used in the synthesis of the various derivatives are summarised in Fig. 1.

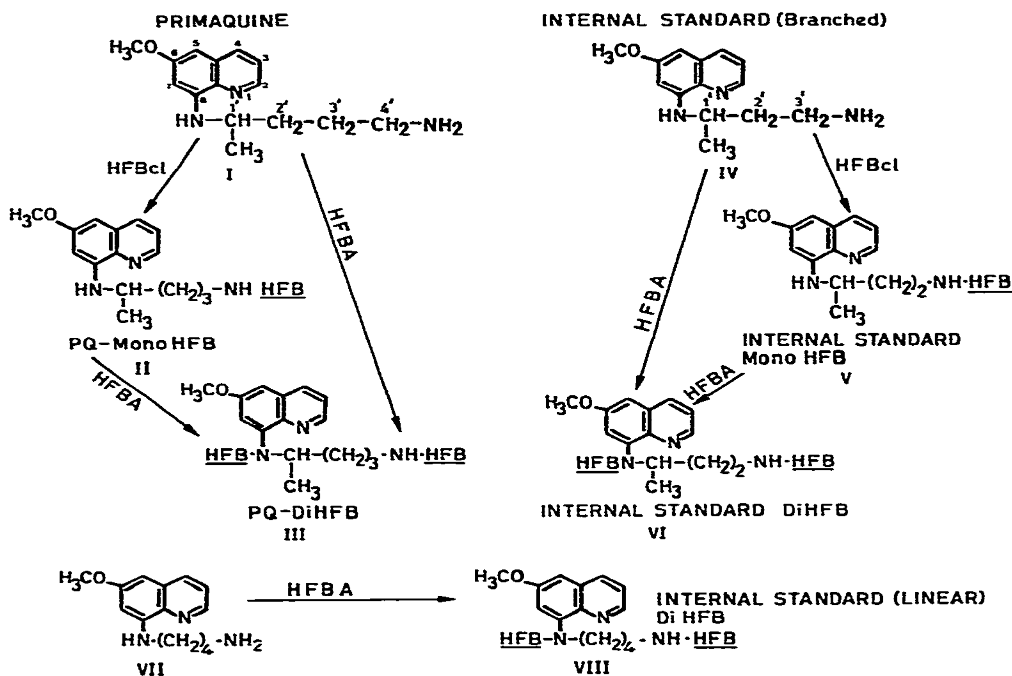

Fig. 1. Summary of reactions used in the synthesis of heptafluorobutyramides of primaquine and internal standard.

Synthesis of monoheptafluorobutyramides II and V

These derivatives were prepared under Schotten—Baumann reaction conditions as described below. The free bases (1 m*M*) of compounds I and IV were liberated from either the diphosphate or the dihydrochloride salts [3, 4] by the addition of 10 ml of 8% aqueous potassium hydroxide at 15°C, to a stirred suspension of the salt in 50 ml of benzene. Then 0.3 ml of heptafluorobutyryl chloride was added dropwise. A second 5-ml lot of 8% potassium hydroxide and 0.2 ml of heptafluorobutyryl chloride were added after a few minutes and the mixture at alkaline pH was stirred at 15°C for 30 min and then at room temperature for 60 min. The benzene layer was separated and the aqueous layer was extracted once with 25 ml of benzene. The pooled benzene layer was washed with water, dried over anhydrous sodium sulphate and filtered. The solvent was removed by rotary evaporation and the residue was purified by chromatography on a 50-g silica gel column equilibrated with hexane—acetone (9:1, v/v). Elution with the above solvent mixture and crystallisation of the gummy residue from hexane yielded the monoHFB derivatives as nice crystalline solids.

A. 8-(4′-heptafluorobutyrylamido-1′-methyl-butylamino)-6-methoxyquinoline or primaquine monoHFB (II). m.p. 88—90°C. Analysis: found C, 50.29; H, 4.77; N, 9.52. $C_{19}H_{20}F_7N_3O_2$ (mol. wt. 455) requires C, 50.11; H, 4.43; N, 9.23. IR: ν_{max}^{KBr} cm^{-1} 3410 (NH); 1700 (amide C = O). UV: λ_{max} 260 (CH_3OH), 287 (CH_3OH + HCl). MS: 455 (M^+), *m/e* 201, 202.

B. 8-(3′-heptafluorobutyrylamido-1′-methyl-propylamino)-6-methoxyquinoline or internal standard monoHFB (V). m.p. 73—75°C. Analysis: found C, 49.03; H, 4.38; N, 9.82. $C_{18}H_{18}F_7N_3O_2$ (mol. wt. 441) requires C, 48.98; H, 4.11; N, 9.52. IR: ν_{max}^{KBr} cm^{-1} 3400 (NH); 1700 (amide C = O). UV: λ_{max} 262 nm (CH_3OH). MS: 441 (M^+), *m/e* 201.

Synthesis of diheptafluorobutyramides III, VI and VIII

A solution of the free base, 1 m*M*, in 10 ml of dry benzene was treated with an excess 1.24 g of heptafluorobutyric anhydride. The mixture which turned an orange colour was refluxed for 90 min with stirring. The solvent was removed first on a rotary evaporator and the excess anhydride was then removed in vacuo. The residue was redissolved in 25 ml of benzene and washed with 10 ml of saturated sodium bicarbonate followed by water. The solvent was removed and the residue was chromatographed on a 40-g silica gel column. Elution with a mixture of cyclohexane—ethyl acetate (8:2) removed the faster-moving by-products. Further elution with cyclohexane—ethyl acetate (6:4), evaporation of these eluates and crystallisation of the residue from hexane—acetone yielded the diheptafluorobutyramides as nice yellow crystals. The above derivatives could also be obtained from the corresponding monoHFBs by heating with HFB anhydride at 100°C for 45 min.

C. 8-(1′,4′-bisheptafluorobutyrylamido-1′-methyl-butylamino)-6-methoxyquinoline or primaquine diHFB (III). m.p. 98—100°C; Analysis: found C, 42.78; H, 3.37; N, 7.28. $C_{23}H_{19}F_{14}N_3O_3$ (mol. wt. 651) requires C, 42.41; H, 2.94; N, 6.45. IR: ν_{max}^{KBr} cm^{-1} 3340 (NH); 1700 (amide C = O). UV: λ_{max} (CH_3OH) 258, 320, 400 nm. MS: 651 (M^+), *m/e* 482, 397, 201.

D. 8-(1′,3′-bisheptafluorobutyrylamido-1′-methyl-propylamino)-6-methoxyquinoline or internal standard diHFB (branched) (VI). m.p. 80—82°C. Analysis: found C, 42.23; H, 3.31; N, 6.95. $C_{22}H_{17}F_{14}N_3O_3$ (mol. wt. 637) requires C, 41.45; H, 2.69; N, 6.59. IR: ν_{max}^{KBr} cm^{-1} 3340 (NH); 1700 (amide C = O). UV: λ_{max} 258, 320, 395 nm. MS: 637 (M^+), *m/e* 468, 397, 201.

E. 8-(1,4′-bisheptafluorobutyrylamido-butylamino)-6-methoxyquinoline or internal standard diHFB (linear) (VIII). m.p. 107—108°C. Analysis: found C, 41.71; H, 2.95. $C_{22}H_{17}F_{14}N_3O_3$ (mol. wt. 637) requires C, 41.45; H, 2.69. UV: λ_{max} (CH_3OH) 265 (broad), 320 (shoulder), 333. MS: (M^+) 637, *m/e* 468, 467.

Method

Step 1. Primaquine diphosphate, 1.76 mg, and 1.8 mg of the internal standard diphosphate (branched) corresponding to 1 mg of free base were dissolved

separately in 10 ml of distilled water and the volume made up to 100 ml with methanol. Internal standard stock solution (100 μl) was diluted to 5 ml with methanol in a volumetric flask to give the working internal standard solution, 20 ng per 100 μl. Volumes of 50, 100, 150 and 200 μl of the primaquine diphosphate stock solution were each diluted to 5 ml with methanol to give solutions containing 10, 20, 30 and 40 ng primaquine in 100 μl. The stock solutions were kept refrigerated and dilutions from stock solutions were made fresh since the diluted solutions were found to be unstable.

Step 2. A 100-μl volume of the diluted internal standard and 100 μl of the primaquine solution containing 10, 20, 30 or 40 ng primaquine were pipetted into a glass-stoppered silanized tube. Then 1.0 ml of blood was added and the tube was gently swirled by hand. This was followed by the addition of 1 ml of 2.5 *N* sodium hydroxide. The tube was swirled and 3 ml of a mixture of cyclohexane—methylene chloride (4:1, v/v) were added. The stopper was sealed with a drop of water and the tube was mounted horizontally on a reciprocal shaker and shaken at full speed for exactly 2 min.

Step 3. The tube was removed from the shaker and centrifuged at 4000 *g* for 10—15 min. The organic layer was transferred to a 4-ml conical glass stoppered tube and concentrated under a gentle stream of nitrogen at 37°C to a volume of approximately 50 μl. The tube was vortexed once or twice to wash the residue down to the tip of the tube.

Step 4. Heptafluorobutyric anhydride (50 μl) was added and the stopper was quickly sealed with a drop of dry benzene. The stopper was sealed with a strip of parafilm and heated at 65—70°C in a water-bath for 30 min.

Step 5. After cooling to room temperature, 1.2 ml of saturated aqueous sodium bicarbonate were added and gently mixed. The tube was allowed to stand for 10—15 min with occasional mixing and the pH checked with indicator paper to ensure complete neutralization.

Step 6. Distilled benzene (200 μl) was added and the tube was mixed at full speed in a vortex mixer for 30 sec and centrifuged.

Step 7. A maximum aliquot of the upper benzene layer was transferred to another tube and a 2- or 3-μl aliquot was injected "on column" into the gas chromatograph in duplicate.

The calibration curve was obtained by extraction of blood spiked with varying amounts of primaquine (from 10 to 40 ng/ml) and a constant amount of internal standard (20 ng/ml of blood). The peak area ratios were calculated by dividing the area of the peak due to primaquine by the area of the peak due to the internal standard. Calibration curves were constructed by plotting peak area ratio as a function of primaquine concentration. Six to eight samples were analysed for each calibration point. This calibration curve was used subsequently to calculate unknown concentrations of primaquine in blood.

RESULTS AND DISCUSSION

Successful quantitative analysis of primaquine at the nanogram range was dependent on the proper selection of an internal standard and the choice of a suitable derivative for electron-capture GC. Several analogues of primaquine were examined before selecting compound II (cf. Fig. 1). This analogue was found to be ideally matched in its extraction, derivatization and GC resolution

behaviour when compared to primaquine. The amine function at 8 and 3′ or 4′ positions of the molecule (cf. Fig. 1, compounds I and IV) was the obvious site for derivatization.

Greaves et al. [2] had reported that attempts to form acyl derivatives with reagents like heptafluorobutyric anhydride and heptafluorobutyrylimidazole resulted in the formation of mixtures of mono- and diacylated products. Therefore, this reaction was investigated in detail to arrive at the optimal conditions for derivatization which would favour the formation of the diheptafluorobutyramides of both primaquine and the internal standard. The synthesis of pure, chemically characterised derivatives of both primaquine and the internal standard helped a great deal in the study of their GC behaviour and in establishing their sensitivity for detection using the electron-capture detector. Fig. 2 illustrates the separation of both mono- and diheptafluorobutyramides of primaquine and internal standard. The diheptafluorobutyramides are approximately 6—8 times more sensitive than the corresponding mono derivatives and possess excellent chromatographic properties. The introduction of fourteen fluorine atoms to both primaquine and the internal standard imparts excellent sensitivity for detection in the sub-nanogram range.

Several solvents and solvent mixtures were tried for the initial extraction of primaquine and the internal standard from blood. A single extraction using the solvent mixture cyclohexane—methylene chloride at a highly alkaline pH (pH

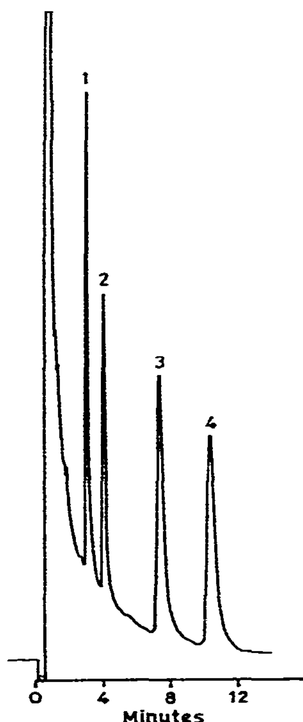

Fig. 2. Chromatogram of synthetic heptafluorobutyramide derivatives of primaquine and internal standard. Peaks: 1 = internal standard monoHFB; 2 = primaquine monoHFB; 3 = internal standard diHFB; 4 = primaquine diHFB.

> 13.0) was found to yield the least complex extract free of extraneous peaks in the primaquine and internal standard diheptafluorobutyramide regions following derivatization and chromatography. The recovery of nanogram quantities of primaquine and internal standard added to blood ranged between 60 and 70% in the extraction step.

The recovery was poor when 2.5 *N* NaOH was replaced by buffers in the alkalinization step. Concentration of the cyclohexane—methylene chloride extract to complete dryness under nitrogen led to severe losses of both primaquine and internal standard. Concentrating the extract to approximately 50 μl with intermittent vortexing to rinse the sides of the tubes resulted in good recoveries of both the compounds. The effect of time, temperature and the amount of heptafluorobutyric anhydride required for maximum yield of the diHFB derivatives was studied by GC using the synthetic derivatives as standards. Plateau conditions were reached in 30 min at 65°C using 50 μl of the anhydride. The overall recoveries of primaquine and the internal standard ranged between 30 and 50% as determined by GC using the standard compounds.

Typical gas chromatograms obtained with the method are shown in Fig. 3. The calibration curve constructed by adding known quantities of primaquine and a constant amount of internal standard to blood was linear from 10 to 40

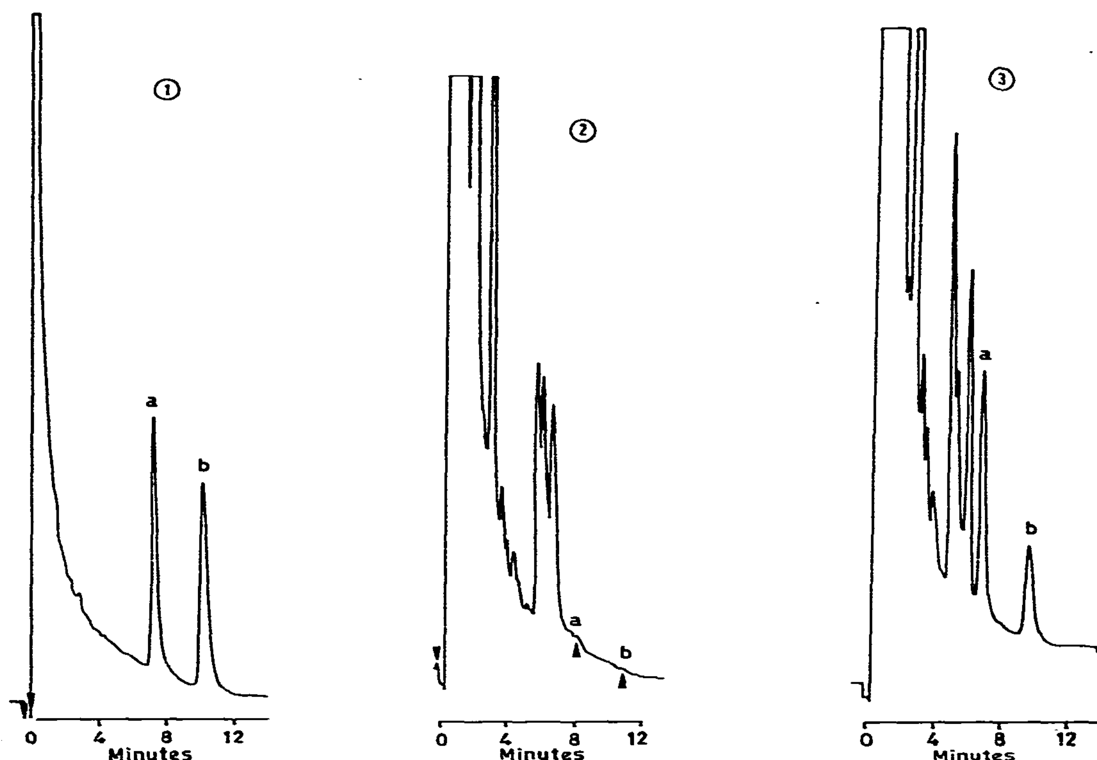

Fig. 3. Examples of chromatograms. (1) 0.2 ng each of internal standard diHFB (a) and primaquine diHFB (b). (2) Human blood blank sample (1 ml). (3) Human blood sample spiked with 8 ng of primaquine and 20 ng of internal standard.

ng/ml primaquine with a minimum detectable concentration of 8 ng/ml, based on a 1-ml sample volume. The equation for the calibration curve from blood was calculated and found to be $y = 0.0521x - 0.01$ with an excellent correlation ($\gamma = 0.999$). Table I shows the results obtained when the above method was applied to spiked blood samples. The results demonstrate good reproducibility of the method to a concentration of primaquine as low as 8 ng/ml of blood.

TABLE I

WITHIN-RUN PRECISION OF THE METHOD APPLIED TO SPIKED HUMAN BLOOD SAMPLES

Amount added (ng/ml)	Amount found (ng/ml) (mean ± S.D., $n = 4$)	Precision/reproducibility (C.V. %)	Mean recovery (%)
8	9.16 ± 0.52	5.67	114.5
15	14.85 ± 0.83	5.6	99
25	25.5 ± 0.25	0.98	102
35	35.75 ± 0.74	2.1	102

The specificity and sensitivity of the described method for pharmacokinetic studies was ascertained by an in vivo experiment in dog. Fig. 4 illustrates the time course of whole-blood levels of primaquine for the first 15 h following the oral administration of an aqueous solution of primaquine diphosphate equal to 0.5 mg/kg body weight of the free base. The maximum concentration of 46 ng/ml was reached in 4 h followed by a rapid decrease to a level of 5.75 ng/ml at 12 h. The levels beyond this point were below the detection limit of the method. The half-life of primaquine in the dog was found to be 2.5 h. The major metabolite of primaquine in man has been identified by Baty et al. [5] as 6-methoxy-8-aminoquinoline. The monoHFB derivative which is formed on acylation with HFB anhydride under the derivatization conditions used in the present method does not elute from the GC column at 230°C, the temperature used in the present method.

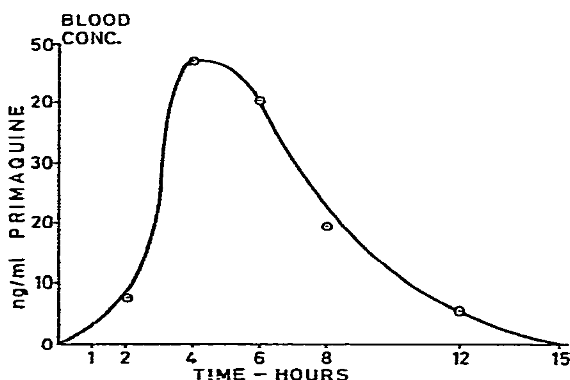

Fig. 4. Concentration of primaquine in dog blood after the administration of an oral dose of 0.5 mg/kg.

The method presented in this paper is simple, fast and extremely sensitive for the quantitative determination of primaquine in biological fluids. Experiments on the application of this technique to study single- and multiple-dose pharmacokinetics of this important antimalarial drug are in progress.

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