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Note

Identification and determination of a carboxylic acid metabolite of chloroquine in human urine by high-performance liquid chromatography

NESBITT D. BROWN*

Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307-5100 (U.S.A.)

BING T. POON

Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307-5100 (U.S.A.)

and

LAWRENCE R. PHILLIPS

Division of Biochemistry and Biophysics, Food and Drug Administration, Bethesda, MD 20892 (U.S.A.)

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Chloroquine, a 4-aminoquinoline, and some of its structural congeners are widely used in the prophylactic and therapeutic treatment of malaria and various connective tissue disorders [1–3]. Results, as indicated from the recovery of metabolic end-products from human subjects receiving the 4-aminoquinoline antimalarials have revealed a series of transformation products [4–7]. Many of the metabolites formed are derived through metabolic schemes shown in Fig. 1.

Recent attempts to elucidate the complex pharmacokinetics of chloroquine have focused on the use of various sensitive and selective liquid chromatographic methods [8,9]. While many of the reported procedures have adequately met the needs of the investigator in determining the metabolic fate of chloroquine in man, one aspect of this degradation process has not been clearly defined. It involves the isolation and identification of the elusive carboxylic acid metabolites of chloroquine.

We report here, a simple, sensitive and specific ion-pair reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of

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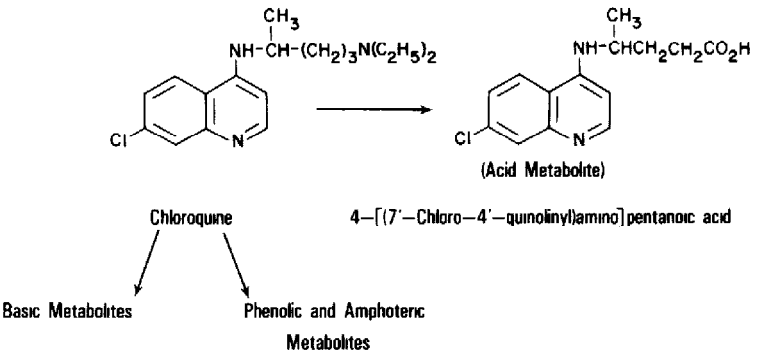

Fig. 1. Metabolic scheme for the metabolism of chloroquine in human subjects, as determined from assayed urine samples. A 300-mg dose of chloroquine diphosphate was administered orally to each subject.

4-[(7'-chloro-4'-quinolinyl)amino]pentanoic acid. The method has been used for measuring this metabolite in urine specimens of volunteers, following the administration of chloroquine.

EXPERIMENTAL*

Apparatus

The method was developed using a Waters Model ALC/GPC-204 liquid chromatograph. The total system consisted of two Model 6000A high-pressure pumps, a Model 660 solvent programmer, a U6K loop injector, a Model 481 LC spectrophotometer, set at 340 nm, a Houston Instrument Omni-Scribe dual-pen recorder and a Columbia Scientific Industries Supergrator-3 integrator.

Reagents

Spectroquality acetonitrile (Burdick and Jackson Labs., Muskegon, MI., U.S.A.) mixed with PIC B-7 reagent (1-heptanesulfonic acid, Waters Assoc., Milford, MA., U.S.A.) was used as the mobile phase. Chloroquine diphosphate and 4-[(7'-chloro-4'-quinolinyl)amino]pentanoic acid were obtained from the Walter Reed Army Institute of Research's Inventory (Washington, DC, U.S.A.). All working standards of 4-[(7'-chloro-4'-quinolinyl)amino]pentanoic acid were prepared from stock solutions, containing 100 ng/μl of the carboxylic acid compound.

Free acid metabolite

A 300 mm$\times$7.8 mm I.D., 10-μm μBondapak C_{18} column (Waters Assoc.) was employed to chromatograph experimental and standard solutions used in the study. The mobile phase consisted of a 0.02 *M* solution of PIC B-7 reagent in water and acetonitrile. PIC B-7 was prepared by dissolving 40 ml of the prepackaged reagent into 460 ml of glass-distilled water. The pH of the solution was 3.4. Acetonitrile–0.02 *M* PIC B-7 (33:67, v/v) was used in an isocratic mode. The

*The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

flow-rate was 2.0 ml/min. Column pressures ranged between 75 and 84 bar. All separations were performed at room temperatures (20–22°C). A 10-μl volume of the acetonitrile extract was injected onto the column through a continuous-flow loop injector. Peak areas were measured by an on-line computing integrator.

n-Propyl ester derivative

To confirm the presence of the carboxylic acid metabolite of chloroquine, propyl 4-[(7′-chloro-4′-quinolinyl)amino]pentanoate was prepared from urine extracts. Similarly, the *n*-propyl ester of 4-[(7′-chloro-4′-quinolinyl)-amino]pentanoic acid was also prepared from the working standards. A rapid esterification procedure was used to prepare the *n*-propyl ester derivatives. The esterification reagent was supplied by Applied Science Labs. (State College, PA, U.S.A.).

Esterification

Boron trifluoride–propanol reagent (5 ml) was added to the dried urine extract and the sample was refluxed in a steam bath for 10 min. The boiled mixture was cooled to room temperature and then mixed with 10 ml of saturated ammonium sulfate. Diethyl ether (5 ml) was added to the reaction mixture and shaken for 2 min in a separatory funnel. The organic layer, containing the derivatized metabolites, was transferred to a 50-ml polypropylene screw-capped tube. Anhydrous sodium sulfate (3 g) was added to the extract. The extract was assayed for the *n*-propyl esters. Separation parameters were similar to those used for the free acid metabolite. A mixture of acetonitrile–0.02 *M* PIC B-7 reagent (75:25, v/v) was used to separate the *n*-propyl ester.

Peak collection

Fractionated aliquots containing the chromatographed peak of the *n*-propyl ester metabolite (retention time 7.32 min) were collected after multiple injections of the experimental samples. The *n*-propyl ester was extracted into an ether–ammonium sulfate mixture. Mass spectrometric determinations were performed on the organic phase extracts. Fast atom bombardment (FAB) mass spectra of the *n*-propyl ester were scanned for the protonated molecular ion $[M+H]^+$ from samples introduced directly into the ion source as colloidal suspensions in a glycerol matrix.

Samples

Urine specimens were collected from two normal subjects. Each volunteer received a single 300-mg dose of chloroquine diphosphate. Urine samples were collected prior to dosing and 12 and 24 h after dosing. Aliquots (25 ml) were removed from the total sample, adjusted to pH 5.5 with concentrated hydrochloric acid and mixed with 50 ml of acetonitrile. Anhydrous sodium sulfate was added to the solution until a two-phase matrix formed. After 2 min of shaking, the organic phase, containing the acidic metabolites, was removed and placed into a 50-ml conical shaped polypropylene tube and blown to dryness with streams of nitrogen gas. The solution was evaporated in a 95°C water bath. Acetonitrile (2 ml) was

added to the dried specimen and mixed thoroughly. Standard solutions of the prepared *n*-propyl ester were treated similarly. A 98% recovery of the propyl 4-[(7′-chloro-4′quinolinyl)amino]pentanoic acid was obtained from spiked urine specimens.

RESULTS AND DISCUSSION

While various methods have been developed and utilized for separating and identifying basic, phenolic and amphoteric metabolites of chloroquine in physiological specimens, the development of simple and specific procedures to separate the acid metabolites of this drug has been lacking. During the past five years, HPLC has proven to be an effective tool for studying the pharmacokinetics of various drugs in humans. The specificity of such methods has helped to shed more light onto the subject involving the metabolism of certain drugs in man. This fact is especially true for chloroquine.

For this study, we utilized a newly developed technique to separate and identify an acid metabolite of chloroquine, which until now has been difficult to accomplish. We now report on the assaying of 4-[(7′-chloro-4′-quinolinyl)amino]-pentanoic acid in urine.

In utilizing the ion-pair reversed-phase HPLC procedure, various unknowns, as well as the acid metabolite of chloroquine, were separated on the semi-preparative column. Analysis for a series of standard solutions (100–1000 ng) produced

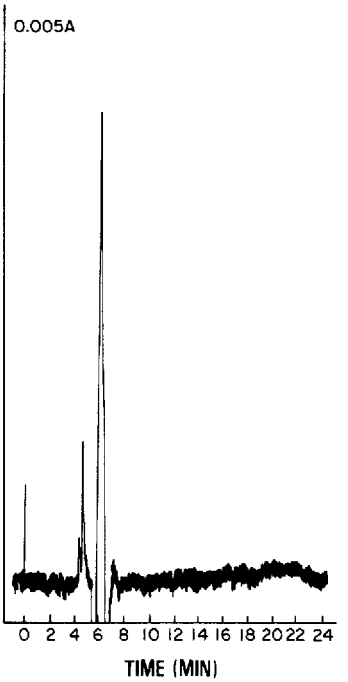

Fig. 2. Chromatogram of the 0-h chloroquine-free urine extract of subject B.

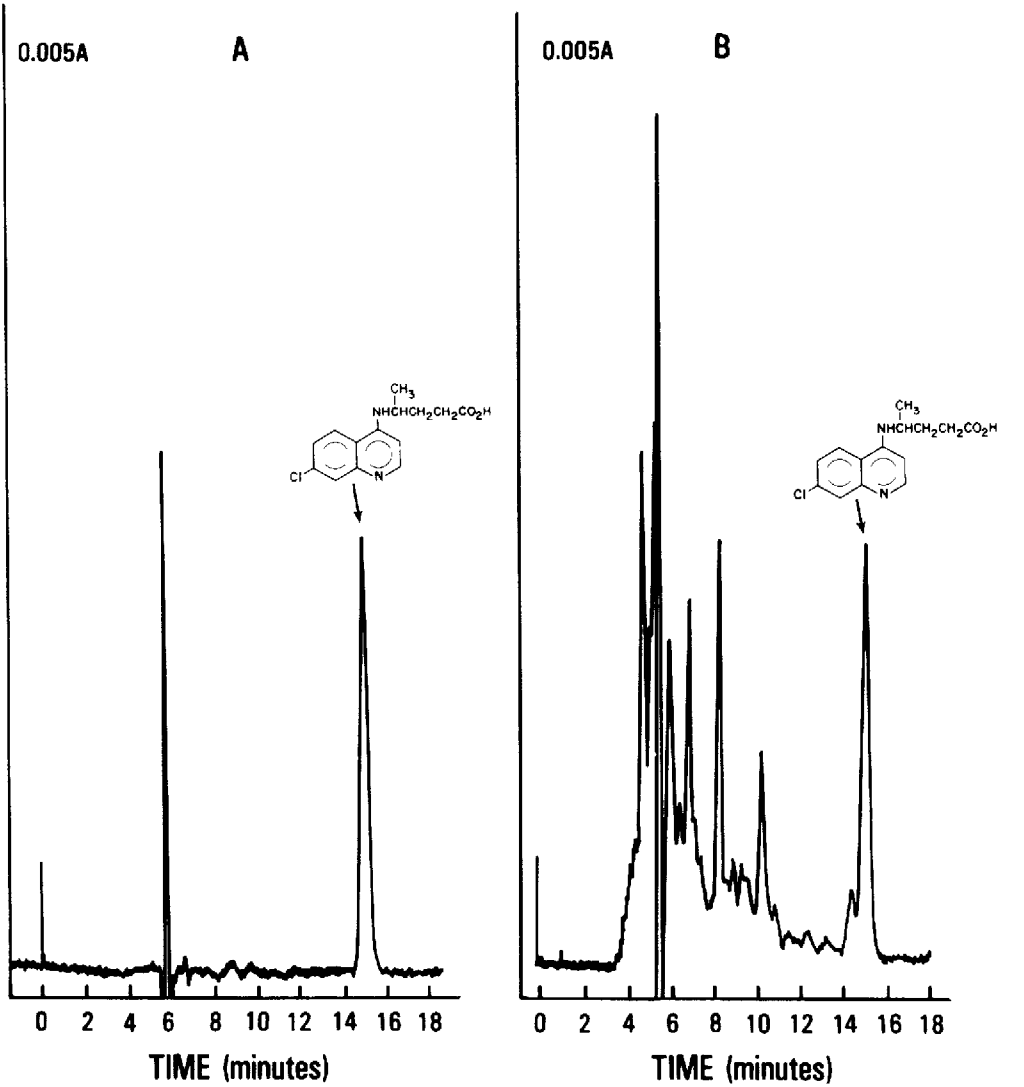

Fig. 3. HPLC profile of (A) a 50-ng standard of 4-[(7′-chloro-4′-quinolinyl)amino]pentanoic acid and (B) a 24-h urine extract of subject B.

linearity for all of the concentrations assayed. The correlation coefficient for 4-[(7′-chloro-4′-quinolinyl)amino]pentanoic acid was 0.992.

We analyzed a series of spiked urine specimens containing the free acid metabolite. Sample concentration of six prepared specimens was 1.50 μg/ml. Data on within-run precision were 1.48 ± 0.02 μg/ml [coefficient of variation (C.V.) 0.37%]. Day-to-day precision data for six spiked urine specimens, as evaluated during a five-day period, were 1.47 ± 0.02 μg/ml (C.V. 0.51%). Lower detection limit for the method was 5 ng on-column with a signal-to-noise ratio of 3:1. Analysis time for the free acid metabolite was 16 min per sample. Chloroquine-free urine (0 h) was chromatographed for subject B (Fig. 2). No peaks were found

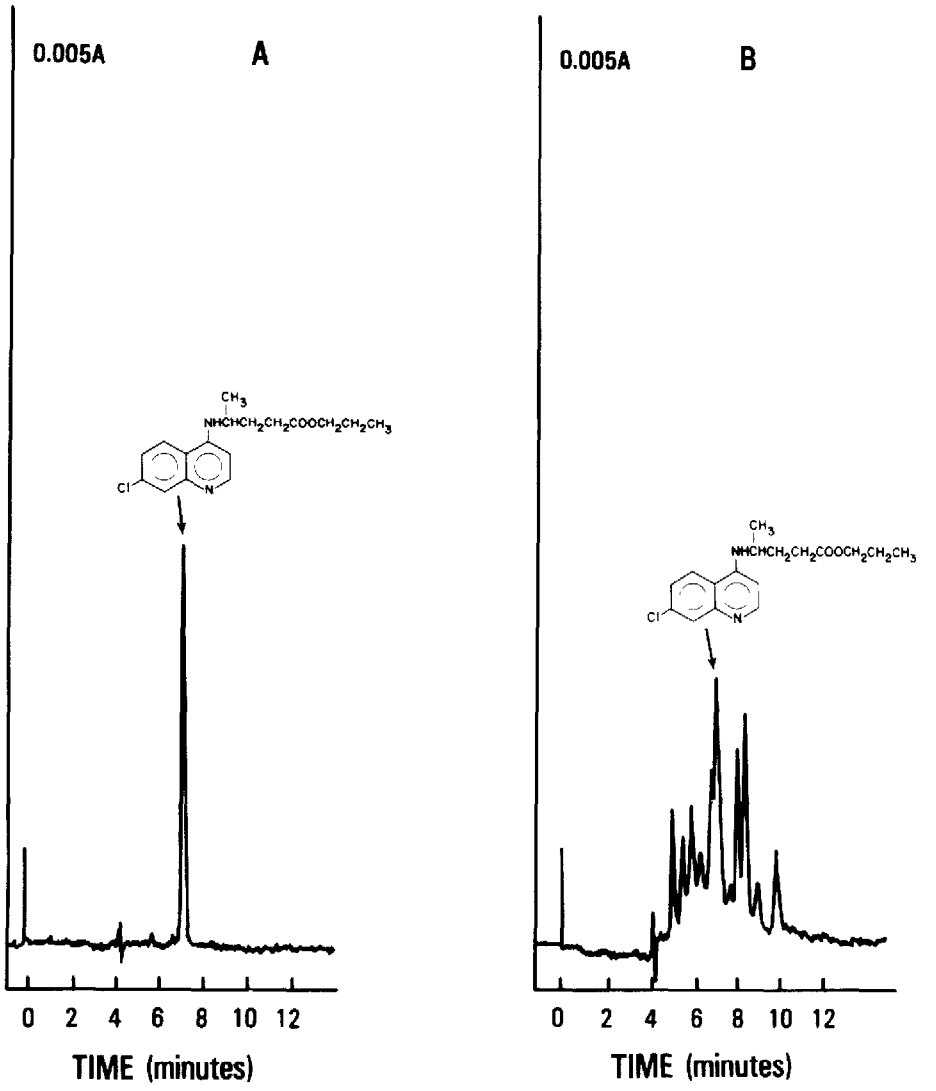

Fig. 4. HPLC profile of (A) a 100-ng standard of propyl 4-[(7′-chloro-4′-quinolinyl)amino]pentanoate, derivatized with boron trifluoride–*n*-propanol, and (B) the esterified 24-h urine specimen from subject B.

which had retention times similar to the carboxylic acid metabolite peak seen in the experimental samples.

The chromatograms shown in Fig. 3 represent the separation of a standard solution of 4-[(7′-chloro-4′-quinolinyl)amino]pentanoic acid and a 24-h urine specimen, collected from a volunteer. For each of the two subjects used in these studies, the acid metabolite was observed in the analyzed urine samples. Approximately 3.1 mg of the metabolite was measured in the total 24-h urine sample of subject A. The calculated amount for subject B was 3.3 mg per 24 h. These recovered amounts represent 1.70 and 1.80%, respectively, of the total urinary excretion output.

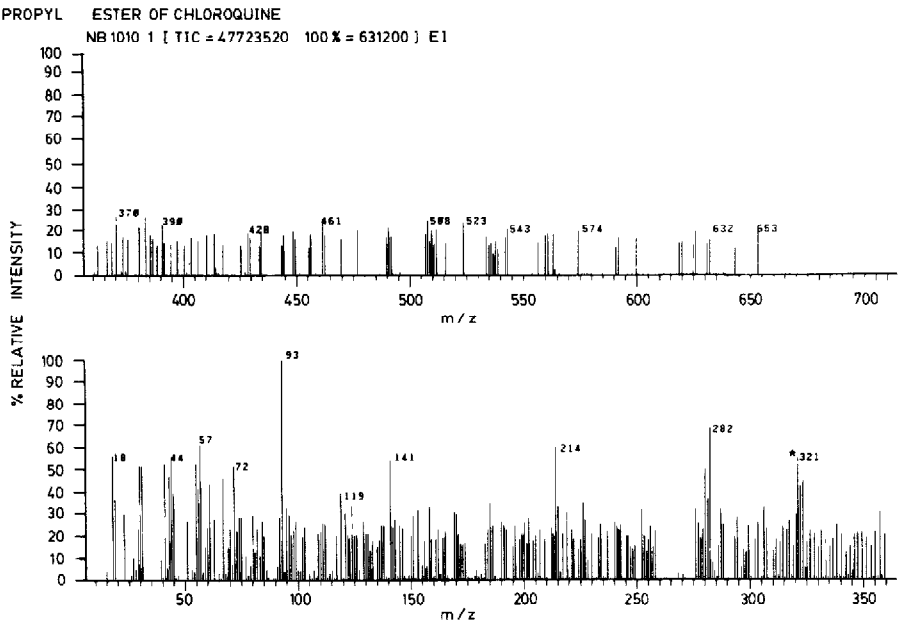

Fig. 5. FAB mass spectra of the *n*-propyl ester derivative, prepared from experimental urine extract. The protonated molecular ion $[M+H]^+$ for the chloroquine metabolite was observed at 321.

To confirm the presence of the free acid metabolite of chloroquine, boron trifluoride–*n*-propanol was used to prepare propyl 4-[(7′-chloro-4′-quinolinyl)amino]pentanoate. The chromatogram shown in Fig. 4A represents a 100-ng standard of the derivatized acid. The chromatogram shown in Fig. 4B is the esterified 24-h urine specimen of subject B.

The *n*-propyl esters of chloroquine prepared in these studies were further analyzed for positive verification of the acid metabolite. The protonated molecular ion (*m/z* 321) for both standards and urine extracts was observed by FAB mass spectrometry. The mass spectra shown in Fig. 5 represent data collected from the derivatized urine specimen of subject B.

From these studies, we were able to separate and identify a long sought-after metabolite of chloroquine in man. In previous studies, isolated metabolites of chloroquine, such as hydroxychloroquine and desethylchloroquine, continued to exhibit anti-malarial properties, when administered to infected mice. The synthesized 4-[(7′-chloro-4′-quinolinyl)amino]pentanoic acid is currently being tested in mice for its anti-malarial therapeutic value.

Further studies are underway to identify the unknown acid metabolites produced in this metabolic pathway.

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REFERENCES

1 C.O. Khoromana, C.C. Campbell, J.J. Wirima and D.L. Heymann, Am. J. Trop. Med. Hyg., 35 (1986) 465.
2 A.M. Bradley-Moore, B.M. Greenwood, A.K. Bradley, B.R. Kirkwood and H.M. Gilles, Ann. Trop. Med. Parasitol., 79 (1985) 575.
3 M. Ostensen, N D. Brown, P.K. Chiang and J. Aarbakke, Eur. J. Clin. Pharmacol., 28 (1985) 357.
4 G. Alván, L. Ekman and B. Lindstöm, J. Chromatogr., 229 (1982) 241.
5 N.D. Brown, B.T. Poon and J.D. Chulay, J. Chromatogr., 229 (1982) 248.
6 N.D. Brown, M.G. Stermer-Cox, B.T. Poon and J.D. Chulay, J. Chromatogr., 309 (1984) 426.
7 N.D. Brown, B.T. Poon and J.D. Chulay, J. Chromatogr., 345 (1985) 209.
8 M.A. Staiger, P. Nguyen-Dinh and F.C. Churchill, II, J. Chromatogr., 225 (1981) 139.
9 Y. Bergqvist and M. Frisk-Holmberg, J Chromatogr., 221 (1980) 119.