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Short Communication

Simultaneous determination of a new antimalarial agent, CDRI compound 80/53, and its metabolite primaquine in serum by high-performance liquid chromatography☆

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ABSTRACT

Compound 80/53 (I) is a new substance being developed as an antimalarial agent. It is unstable in acidic conditions where it is converted into primaquine. A high-performance liquid chromatographic assay for simultaneous determination in serum of I and primaquine has been developed. Conditions were optimized to minimize the conversion of I into primaquine. The method includes extraction of the unchanged compound and primaquine from serum samples with hexane–2-propanol (pH > 8). Separation was accomplished by reversed-phase chromatography on a C_{18} column with acetonitrile–tetrahydrofuran–phosphate buffer. The recoveries of I and primaquine were always greater than 70%. No interference was observed in extracts obtained from drug-free serum. The detector response was linear with concentrations of I and the metabolite in the ranges 25–400 and 10–180 ng/ml, respectively, and the within-day precision (coefficient of variation) remained less than 13.7% for I and 12.5% for primaquine. The method is suitable for the determination of concentration–time profiles of I and primaquine in human serum.

INTRODUCTION

The CDRI compound 80/53, N′-3′-(acetyl-4′,5′-dihydro-2′-furanyl)-N-(6-methoxy-8-quinolinyl)-1,4-pentanediamine (I) (Fig. 1) is a potent new antimalarial agent synthesized as the prodrug of primaquine [1,2]. Compound I shows radical curative and causal prophylactic activities against sporozite-induced *Plasmodium cyanomolgi* infection in rhesus monkeys [3,4]. It is safer than primaquine (II) and causes only one third as much methaemoglobinaemia [5]. It is also safe in subacute toxicity studies in rats and rhesus monkeys and has no teratogenic action [6]. Currently it is under Phase I clinical studies.

To support the development of I as a candidate antimalarial agent, a rapid, accurate and precise assay of the serum concentrations of I and its active metabolite II in animals and humans is essential. Several methods have been reported for the quantitation of II, using gas chromatography (GC) [7,8] and high-performance liquid chromatography (HPLC) [8–11]. However, there is no published assay technique for I. Moreover, owing to the instability [12] of I in aqueous systems below pH 7, leading to its conversion into II, it was of interest to develop an assay method for

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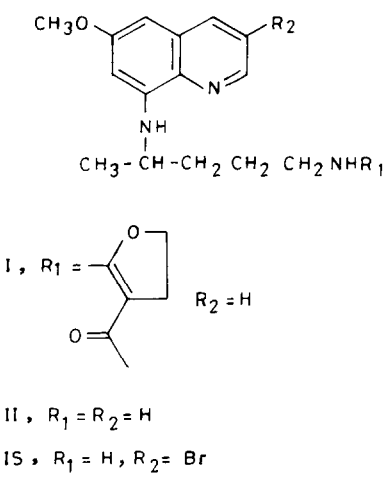

Fig. 1. Structures of CDRI compound 80/53 (I), primaquine (II) and the internal standard (I.S.).

their simultaneous determination in biological samples where the conversion of I into II during storage or assay is minimized.

EXPERIMENTAL

HPLC apparatus and chromatographic conditions

The HPLC system consisted of a Model PU 4015 (Pye Unicam, Cambridge, UK), a Model 7125 injector with a 50-μl fixed loop (Rheodyne, Berkeley, CA, USA), a Uvikon 730S LC variable-wavelength UV detector (Kontron, Zurich, Switzerland) set at 269 nm, and a C_{18} (5 μm) cartridge column (100 mm × 4.6 mm I.D.) preceded by a C_{18} precolumn (30 mm × 4.6 mm I.D.) (Pierce, Rockford, IL, USA). A diode-array detector (Waters Model 991, Milford, MA, USA) was connected after the UV detector in series to record the UV spectra of eluting peaks. Chromatograms were recorded and integrated by Nelson software (Nelson Analytical, Cupertino, CA, USA) on a PC/XT computer. A Model SVC-200H Savant Speed-Vac concentrator (Savant Instruments, New York, NY, USA) was used to evaporate the organic solvent during extraction.

Reagents and solvents

Compound I was obtained from the Chemical Technology Division of this Institute and further purified by repeated crystallization to obtain pure standard reference. The internal standard (I.S.), 3-bromoprimaquine diphosphate, was obtained as a gift from Prof. James D. McChesney of the University of Mississippi, USA. Compound I and the I.S. were assayed at 99% purity. Primaquine diphosphate (>99% pure) was obtained from Aldrich (St. Louis, MO, USA). Dipotassium hydrogenorthophosphate, potassium dihydrogenorthophosphate and potassium hydroxide (S.D. Fine-Chem, Bombay, India), and orthophosphoric acid (Glaxo, Bombay, India) were of analytical grade. HPLC-grade acetonitrile, tetrahydrofuran, hexane and 2-propanol were procured from Spectrochem (Bombay, India). N,N′-Dimethyloctylamine (DMOA) was prepared from octylamine in this laboratory [13]. Triply distilled water from an all-quartz apparatus was prepared and used as a solvent.

The extraction solvent was prepared by adding 1 ml/l DMOA to hexane–2-propanol (97:3). The reconstituting solvent was prepared by adding 1 ml/l DMOA to acetonitrile–0.05 *M* potassium dihydrogenorthophosphate (60:40) adjusted to pH 7.5 with orthophosphoric acid.

Chromatographic conditions

The mobile phase was acetonitrile–0.05 *M* dipotassium hydrogenorthophosphate (adjusted to pH 6 with 20% orthophosphoric acid)–tetrahydrofuran (60:39:1), containing 0.5 ml/l DMOA. It was filtered and degassed before use. Chromatography was performed at ambient temperature. The mobile phase flow-rate was 0.7 ml/min.

Stock and standard solution preparation

A stock solution containing 160 μg/ml was prepared by dissolving 8 mg in 50 ml of acetonitrile with 50 μl of DMOA. Stock solutions of II and the I.S. were prepared by dissolving 2.81 mg of primaquine diphosphate (equivalent to 1.6 mg of II) and 5 mg of the I.S. in 50 ml of triply distilled water in separate volumetric flasks. Subsequent dilutions of I with acetonitrile, II and the I.S. with triply distilled water were made from stock

solutions so that the desired amounts (50–1000 μl) of I, II and the I.S. could be conveniently delivered with automatic pipettes.

Extraction

The extraction was carried out in 10-ml glass culture tubes pretreated with extraction solvent containing DMOA to prevent conversion of I into II. A serum sample (0.5 ml) was mixed with 50 μl of the I.S. (5 μg/ml) and 50 μl of 1.0 *M* potassium hydroxide, and extracted three times with 2.5 ml of extraction solvent by vigorous vortex-mixing for 1 min. The mixture was centrifuged for 10 min (1000 *g*) at 4°C, the aqueous layer was frozen in liquid nitrogen and organic extract was transferred to a clean 10-ml conical centrifuge tube (rinsed with extraction solvent). The combined organic layers were concentrated under reduced pressure using a Speed-Vac concentrator. The residue was dissolved in 200 μl of reconstituting solvent and injected into the HPLC system.

Extraction efficiency

Serum was purchased from a local blood bank to generate a drug-free serum pool. To determine the extraction efficiency, a standard solution containing both I and II was added to aliquots (0.5 ml) of serum to achieve 25, 100 and 400 ng/ml I and 10, 40 and 160 ng/ml II, with a constant I.S. concentration of 500 ng/ml. Samples were processed as outlined above and final extracts were used for HPLC analysis. Because all phase transfers were quantitative, the recoveries of I, II and the I.S. were calculated by comparing the peak height of these compounds from extracted samples with those obtained from the analysis of equivalent amounts of the respective compounds injected directly.

Detector response

The linearity of the detector response was investigated after the extraction of serum (0.5 ml) spiked in replicates ($n = 5$) to contain 25, 50, 100, 200 and 400 ng/ml I and 10, 20, 40, 80 and 160 ng/ml II and a constant 500 ng/ml concentration of the I.S. Peak-height ratios (I/I.S. and II/I.S.) *versus* respective the concentrations of I and II were evaluated for linearity by least-squares analysis after injection of sample extracts.

Accuracy and precision

Intra- and inter-day precision and accuracy values were determined by analysing the replicate ($n = 5$) samples of pooled serum fortified with I and II at three concentration levels (low, medium and high). Aliquots of 0.5 ml of serum, spiked with 25, 200 and 400 ng/ml I and 10, 40 and 160 ng/ml II, were processed as described.

Selectivity

Drug-free human serum was routinely analysed as above, and the resultant chromatograms were examined for the presence of endogenous coextractants that could possibly interfere with the measurement of I, II or I.S.

Stability

Aliquots (0.5 ml) of serum samples fortified with 100 ng/ml I were stored for six months at −80°C. Replicates ($n = 2$) were processed and analysed by HPLC on different days during this period with freshly spiked samples to determine the *in vitro* conversion of I into primaquine on storage. Stock standards of I stored at 4°C were also analysed frequently over a period of six months.

RESULTS AND DISCUSSIONS

Chromatography

A C_{18} column was used to separate I, II and the I.S. An increase in the pH of the mobile phase results in peak broadening and decreased resolution of I and II. Although a low pH is indicated for better separation of I, II and I.S., low pH conditions were unsuitable owing to the instability of I. Thus, selectivity for the separation of II from the parent compound without any decomposition was best achieved at pH 6.0. This is the pH of the phosphate buffer measured by a combined glass electrode, and calibrated with aqueous buffers of pH 4.0 and 7.0. The salt concentration in the buffer also influenced the separation of I, II and the I.S. Replacement of the buffer with

triply distilled water sharpens the peak of I, but the elution of II was delayed (31.5 min). An increase in the salt concentration of the buffer shortens the retention time of all three compounds. Best results were obtained with 0.05 *M* potassium dihydrogenphosphate. Similarly, an increase in the proportion of acetonitrile decreased the retention times of I, II and the I.S., whereas a decrease caused peak broadening as well as increased retention times.

Addition of amines

Degradation of I into II on during the processing of serum samples was the major problem encountered during the development of this assay. It was observed that the addition of various amines retarded the conversion of I into II at ambient temperature. Various amines, such as ammonia, diethylamine, triethylamine and DMOA, were tested. The addition of DMOA (1 ml/l) to the extraction solvent and to the reconstitution solvent prepared with the pH 7 buffer resulted in the lowest rate of conversion. The use of buffers of lower pH in the reconstitution solvent increases the degradation of I, hence we decided to add DMOA to the mobile phase, the reconstituting solvent and the extraction solvent. The glassware used for the extraction and reconstitution of residue were also pre-rinsed with extraction solvent containing DMOA.

The serum extracts reconstituted in reconstitution solvent were stable for up to 4 h at 4°C. However, 1–2% of II was detected in the reconstituted extracts stored at 4°C and analysed after 24 h. It was also essential to reconstitute the dried extracts immediately after evaporation: leaving them longer increased the amount of conversion of I into II.

Selectivity and specificity

Fig. 2 shows typical chromatograms of an extract of drug-free human serum (A), drug-free serum spiked with 50 ng/ml I, 10 ng/ml II and 500 ng/ml I.S. (B), and a mixed standard solution of I, II and the I.S. in the mobile phase (C). The retention times of I, II and the I.S. were *ca.* 5.7, 3.9 and 6.4 min, respectively. No interfering

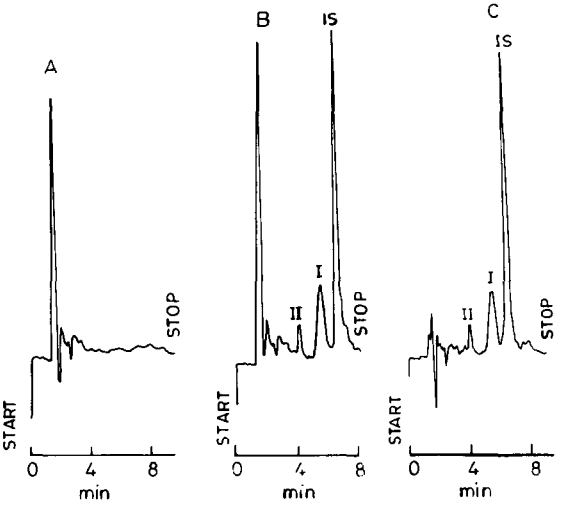

Fig. 2. Chromatograms of (A) drug-free serum, (B) serum spiked with 50 ng/ml compound 80/53, 10 ng/ml primaquine and 500 ng/ml I.S., and (C) a standard solution containing 100 ng/ml compound 80/53, 20 ng/ml primaquine and 1000 ng/ml I.S.

peaks were detected in the control human serum samples. Serum components eluting prior to and after I, II and the I.S. did not interfere with the analysis, and baseline separation was achieved between I, II and the I.S. under the chromatographic conditions described. Chromatographic peaks of I and II in serum samples were characterized by recording 200–400 nm spectra with the UV–photodiode array detector, and comparing them with those of authentic reference standards by the spectral overlay technique. The purity of these peaks was confirmed on the diode array using peak purity parameters [14].

Detector linearity

Six-point standard curves were generated following the extraction of fortified serum samples of I and II in the ranges 25–400 and 10–160 ng/ml, respectively. Least-squares analysis (r = 0.9991) of the peak-height ratios of I to the I.S. and II to the I.S. was done against the respective concentrations. The equation of the best-fit line was used to quantitate the concentrations of I and II in fortified QC serum samples.

TABLE I

PRECISION AND ACCURACY FOR COMPOUNDS I AND II IN SPIKED SERUM SAMPLES

Concentration added (ng/ml)	Within-day			Day-to-day		
	Mean found (ng/ml)	R.S.D.[a] (n = 5) (%)	Relative error (%)	Mean found (ng/ml)	R.S.D.[a] (n = 6) (%)	Relative error (%)
Compound I						
25	23.36	13.7	−6.56	26.2	14.8	+4.8
100	103.56	4.0	+3.56	98.3	10.2	−1.7
400	401.98	3.5	+0.5	397.2	2.7	−0.7
Compound II						
10	9.54	12.5	−4.6	10.8	15.3	+8.0
40	42.84	6.9	+7.1	42.4	8.6	+6.0
160	169.35	4.3	+5.84	166.32	4.2	+3.95

[a] R.S.D. = (S.D./mean) × 100%.

Extraction efficiency

The extraction efficiency was determined for I at 25, 100 and 200 ng/ml, for II at 10, 40 and 160 ng/ml and for the I.S. at 500 ng/ml in serum. The extraction efficiencies (mean ± R.S.D., n = 12) were 50 ± 12.3% for I, 58 ± 13.2% for II and 53 ± 8.6% for the I.S.

Stability of I

Stock solutions of I prepared in acetonitrile containing 1 ml/l DMOA were stable for at least six months. However, the analysis of dilutions of I prepared in reconstitution solvent indicated 1–2% conversion of I into II after 24-h storage at 4°C. Serum samples spiked with 400 ng/ml I did not show any appreciable (less than 2%) conversion of I into II on frequent analysis during six months storage at −80°C.

Assay validation

Method validation was defined in terms of within-day and day-to-day precision. The within-day precision was checked by analysing replicate (n = 5) serum samples spiked with I and II at three concentrations. The relative standard deviation (R.S.D.) was used as a measure of the precision, and the relative difference between the found and added amounts as a measure of accuracy (Table I).

For determining the day-to-day precision and accuracy, serum samples spiked at three concentrations with I and II were treated as unknown and were analysed on five different days. The concentrations of these samples were calculated from the calibration graphs plotted simultaneously each day (Table I).

Limit of quantitation

The lower limit of quantitation in human serum was estimated at 25 ng/ml for I and 10 ng/ml for II. Lower concentrations could still be quantitated with an R.S.D. greater than 15%.

Application of method in clinical pharmacokinetics

The assay method described here was used to obtain single-dose pharmacokinetics of I in healthy volunteers. The method was sensitive enough to follow I and II for up to 24 h after a single 50-mg oral dose. The results of this study will be published elsewhere.

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