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Short Communication

High-performance liquid chromatographic method for the determination of a candidate 8-aminoquinoline antimalarial drug (WR 242511) using oxidative electrochemical detection

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ABSTRACT

WR 242511 (or I) is a new compound of the 8-aminoquinoline class designed to replace primaquine for the treatment of malaria. In order to perform preclinical and clinical testing, an assay was needed to determine drug levels in plasma samples. A simple and reliable reversed-phase high-performance liquid chromatographic (HPLC) method for the measurement of I in plasma using oxidative electrochemical detection is described. A 250-μl plasma sample containing WR 256408 (or II) as internal standard was extracted with *tert.*-butyl methyl ether–2-propanol. A 25-μl aliquot of the extractant was used for HPLC analysis. The mobile phase was 50:50 acetonitrile–sodium acetate (50 m*M*, pH 6) with 1 m*M* EDTA. Compounds I and II were separated within 10 min. The limit of detection for I was 10 ng/ml (plasma) with a recovery around 72%. The method was validated in a dog experiment where levels were followed for 48 h. The method is sensitive and robust and can be used for routine drug analysis during pharmacokinetic studies.

INTRODUCTION

Malaria affects most of the equatorial regions of the world. Radical cure and prophylaxis for vivax and ovale malaria is with the drug primaquine, an 8-aminoquinoline. It is the only significant clinical drug of this class [1]. It also has several toxicities which make it less than ideal for its role as a curative agent. These toxicities include intravascular hemolysis in patients with glucose-6-phosphate dehydrogenase deficiency, gastrointestinal disturbance, methemoglobinemia and leukopenia. The development of other drugs to replace primaquine is of critical importance [2]. Another compound of the 8-aminoquinoline class that has been shown to be superior to primaquine and several other 8-aminoquinolines in a rhesus monkey model of radical cure is 8-[(4-amino-1-methylbutyl)amino]-5-(1-hexyloxy)-6-methoxy-4-methylquinoline (DL)-tartrate (WR 242511, I, Fig. 1) [3]. In order to support the preclinical development of this drug in animal efficacy, toxicology and pharmacokinetic studies, a

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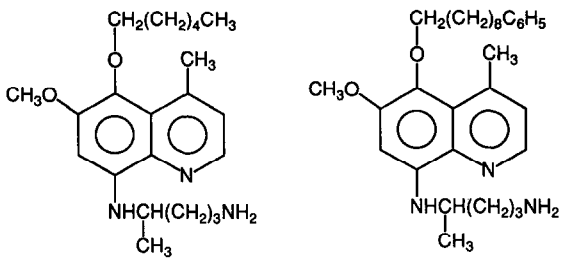

Fig. 1. Structures of WR 242511 (I) and WR 256408 (II).

sensitive, accurate and precise assay was needed to measure I in low-volume plasma samples. High-performance liquid chromatography (HPLC) with UV and with fluorescence detection have been reported for compounds of the 8-aminoquinoline class [4,5], but methods that have employed electrochemical detection have shown equal or better sensitivity [6,7]. A one step liquid–liquid extraction combined with oxidative electrochemical detection is described.

EXPERIMENTAL

Chemicals

Compound I and WR 256408 (II), 8-[(4-amino-1-methylbutyl)amino]-5-(9-hydroxynonylbenzene)-6-methoxy-4-methylquinoline (DL)-tartrate (Fig. 1), were obtained from Ash Stevens (Detroit, MI, USA) and Nodiff/Franklin Research Center (Philadelphia, PA, USA), respectively. Acetonitrile, *tert.*-butyl methyl ether, 2-propanol, glacial acetic acid, disodium EDTA and sodium acetate were obtained commercially and were of HPLC grade. [4]-^{14}C-I with a specific acitivity of 39.9 μCi/mg was obtained from the Research Triangle Institute (Research Triangle Park, NC, USA). Aquasol-2 scintillation fluid was obtained from Dupont/NEN Research Products (Boston, MA, USA). Borosilicate scintillation vials (20 ml) were obtained from Kimble (Vineland, NJ, USA). Outdated human plasma was obtained from the Walter Reed Army Medical Center Blood Bank. Polyethylene glycol was obtained from Sigma (St. Louis, MO, USA).

Apparatus

All blood and plasma samples were stored in 1.5-ml polypropylene microcentrifuge tubes. Extractions were performed in 1.5-ml polypropylene microcentrifuge tubes and centrifugation was performed in an Eppendorf 5413 centrifuge. The HPLC system consisted of a Waters WISP 710B autosampler, a Waters 6000A HPLC pump, a Thompson 5-μm, 30 mm × 4.6 mm I.D. cyano precolumn, a Waters μBondapak, 150 mm × 3.9 mm I.D. cyano column, a BAS LC-4B electrochemical detector in the oxidative mode and a Waters 820 controller. The drug and internal standard were detected using a glassy carbon electrode and an applied voltage of 0.5 V (*versus* Ag/AgCl reference electrode). The signal was monitored at 10 nA full scale. A Packard Tri-Carb 2500TR liquid scintillation counter with automatic quench and chemiluminescence correction was used for ^{14}C radioactive recovery studies.

Extraction

Plasma samples (0.25 ml) were extracted with a 3:1 *tert.*-butyl methyl ether–2-propanol mixture (0.25 ml) by vortex-mixing for 10 s and centrifuging at ambient temperature for 3 min. The organic layer was pipetted off and placed into micro WISP vials.

Standard curve

Stock solutions of I (0.1 mg/ml) and II (0.25 mg/ml) in methanol were kept in light-protective containers at 4°C. Prior to its use as an internal standard, II was diluted 7:1 with methanol (final concentration 31.25 ng/μl). The plasma samples used as standards were prepared by adding 60 μl of the stock solution of I (in methanol) to 3 ml of blank human plasma and by diluting that solution 3:1 with blank human plasma to give solutions of 2 and 0.5 μg/ml in plasma, respectively. Serial dilutions were made to obtain concentrations between 10 and 2000 ng/ml. II (10 μl or 312.5 ng) was added as internal standard directly into the 0.25-ml plasma sample. Standard curves were run for each validation series and for each animal. Validation samples (n = 4) of 1000 and

50 ng/ml I were run for the dog experiment (described below).

Liquid chromatographic procedures

The plasma samples were extracted, the organic layer was pipetted into micro WISP vials and 25 μl were injected on column. The mobile phase consisted of 50:50 (v/v) acetonitrile–50 m*M* sodium acetate and 1 m*M* disodium EDTA (pH 6.0) running at 2 ml/min. Mobile phase was recirculated. Each sample run lasted 10 min.

Quantitation

The concentration of I in extracted experimental samples was determined by comparison of the assayed peak-height ratios to those of a standard curve generated with each run and treated exactly as the unknows. Standard curves were derived by non-weighted least-squares linear regression and fit to the equation $y = mx + b$, where m equals slope and b equals intercept. For each run the standard curve was divided into high and low ranges. The high range encompassed all standard concentrations (10–2000 ng/ml) and was used to calculate the concentrations in samples where the peak-height ratio exceeded that of the 250 ng/ml standard. Samples where the peak-height ratio was less than or equal to the 250 ng/ml standard were compared to the low-range standard curve obtained by fitting the standard concentration data from 10 to 250 ng/ml.

In vivo experiment

Animals used in these studies were handled and housed in accordance with National Institutes of Health guidelines 85-23 for the use and care of animals. The drug was administered as a polyethylene glycol (average molecular mass 200) solution (10 mg/ml). The dog was kept without food but with access to water from 12 h prior to the study to 4 h post dosing and received 3.5 mg/kg I as a single intravenous dose. Blood samples were collected at the following times: 0, 3, 5, 10, 15, 20, 30 min and at 1, 2, 3, 4, 5, 6, 7, 8, 12, 24, 30, 48 and 72 h post dosing. Blood samples (1 ml) were centrifuged at ambient temperature for 5 min in a microcentrifuge and the plasma was separated and frozen at −20°C until analyzed. The plasma samples were thawed and 0.25 ml was extracted and prepared as previously described.

RESULTS

Retention times

Typical chromatograms of blank human plasma, spiked human plasma and dog plasma fol-

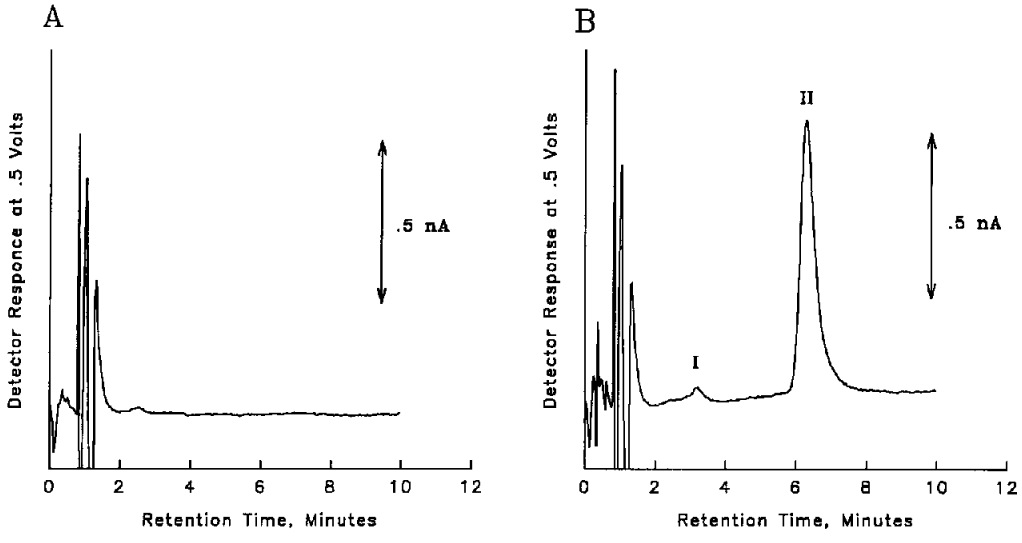

Fig. 2. HPLC of extracts from (A) blank human plasma and (B) human plasma (0.25 ml) spiked with 2.5 ng of compound I and 312.5 ng of compound II.

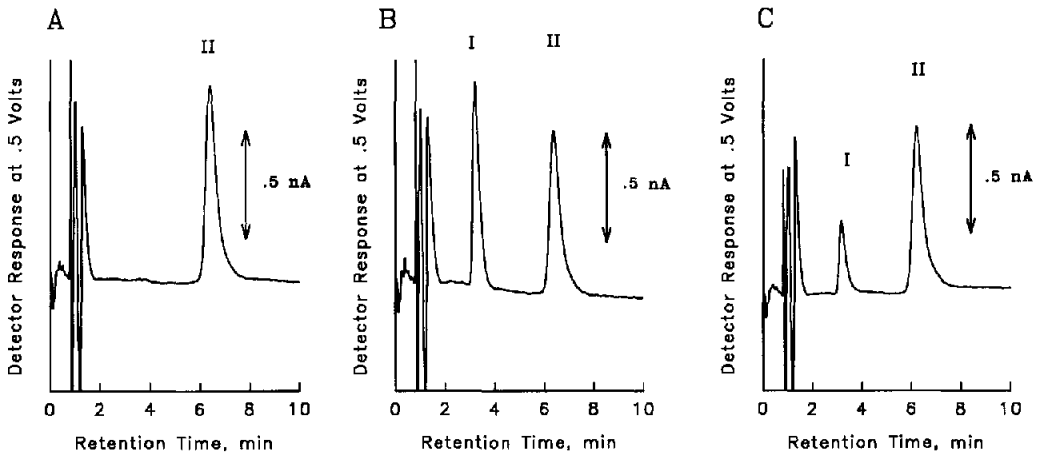

Fig. 3. HPLC of extracts from (A) blank canine plasma prior to dosing, (B) canine plasma 3 min after dosing with 3.5 mg/kg, intravenously (384 ng/ml was detected) and (C) canine plasma 24 h after dosing with 3.5 mg/kg, intravenously (180 ng/ml was detected). Compound II represents 312.5 ng of internal standard.

lowing oral administration are shown in Figs. 2 and 3. The internal standard (II) elutes at 6.25 min and I at 3.1 min. The blank human and dog plasma demonstrate the lack of interfering peaks within the vicinity of both the drug and the internal standard peaks.

Extraction efficiency

Recovery of I and II was measured by comparison of peak heights between compound injected directly on the column and after extraction from plasma. The recovery of I (mean ± S.D.) was 72 ± 9% at 100 ng/ml from a 0.25-ml sample (n = 6). Recovery for II was 100% at 312.5 ng/ml from a 0.25-ml sample (n = 6). Stability of I was examined by measuring a stock plasma sample of 2 μg/ml kept at −20°C for sixty days without loss of peak response.

TABLE I

PRECISION AND ACCURACY OF THE ASSAY OF I

Theoretical concentration (ng/ml)	Day	n	Analytical concentration (ng/ml)	C.V. (%)		Error[a] (absolute value) (%)
				Within-day	Between-day	
1000	1	6	1011 ± 88	8.68	0.83	1.11
	2	6	1019 ± 58	5.71		1.90
	3	6	1031 ± 23	2.23		3.14
	4	6	1016 ± 54	5.36		1.61
100	1	6	107.9 ± 4.7	4.37	1.95	7.90
	2	6	106.1 ± 6.9	6.48		6.13
	3	6	111.0 ± 7.9	7.07		11.0
	4	6	107.2 ± 10.9	10.2		7.17
25	1	6	24.2 ± 1.3	5.29	5.46	3.38
	2	6	23.8 ± 1.7	6.95		4.93
	3	6	23.6 ± 4.9	21		5.73
	4	5	26.5 ± 4.9	18.5		6.05

[a] Error is defined as (measured concentration − theoretical concentration) × 100/theoretical concentration.

Quantitation

A typical high-range standard curve is represented by the equation $y = 0.004\ (\pm 0.001)x + 0.075(\pm 0.059)$, $r^2 = 0.994$, where the numbers in parentheses are the standard errors. A representative low-range curve is described by the equation $y = 0.004(\pm 0.001)x + 0.002(\pm 0.002)$, $r^2 = 0.999$.

Method validation

Data defining the precision and accuracy of the determination of I in the range 10–2000 ng/ml are given in Table I. Accuracy of the method was determined by analyzing plasma samples spiked with 1000, 100 and 25 ng/ml as unknowns as shown in Table I. Precision of the method over the 25–1000 ng/ml range was determined by the analysis of replicate spiked samples done over four days as shown in Table I.

Dog experiment

A pilot study using an intravenous formulation was performed to assess the utility of the analytical method for pharmacokinetic studies. Peak drug concentrations of 375 ng/ml were found immediately following the intravenous bolus injection. Plasma drug concentrations were found up to 48 h after dosing which could allow for adequate drug monitoring for future pharmacokinetic studies. A drug concentration–time profile is given in Fig. 4.

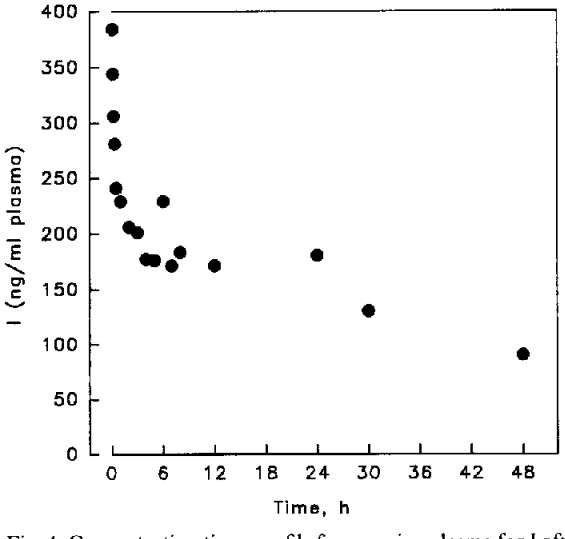

Fig. 4. Concentration time–profile from canine plasma for I after a 3.5 mg/kg dose given intravenously.

DISCUSSION

The method described in this report represents a precise and accurate method for a new 8-aminoquinoline antimalarial drug in dog plasma. The method's limit of detection is 10 ng/ml from a 250-μl plasma sample corresponding to 7 ng/ml free base. It will be used in pharmacokinetic studies designed to describe drug disposition over 48 h.

Prior to developing this assay using electrochemical detection, several detection systems were tried. UV absorption showed an absorbance peak at 240 nm but the limit of detection was only 250 ng/ml I. Fluorescence detection was not found to be useful for I since it demonstrated broad and non-specific absorption and emission bands.

Several extraction methods were tried for this compound before using the one step liquid–liquid extraction. Protein precipitation with acetonitrile, methanol or ethanol followed by direct analysis of the supernatant gave good recovery (>80% by ^{14}C mass balance), but had background noise and interfering peaks precluding its use. The method of direct precipitation also resulted in increasing column backpressure after repeated injections, probably due to protein build-up. Solid-phase extraction on cyano, C_{18}, C_2 and phenyl columns all demonstrated poor recovery (<25% by ^{14}C mass balance) with a variety of eluents. Adequate recovery of radioactivity was obtained by using a mobile phase in the pH 2–4 range as the elution buffer, but the lack of any chromatographic peaks suggested a pH-dependent reaction altering the compound. Liquid–liquid extraction with *tert.*-butyl methyl ether and 2-propanol demonstrated good recovery and a clean baseline without interfering peaks. Variability in recovery of replicate samples was excessive (C.V. >20%) when the organ-

ic layer was dried under nitrogen in silanized tubes and reconstituted in mobile phase. Direct injection of the organic fraction decreased variability in drug recovery as compared to drying and reconstituting with mobile phase.

The addition of disodium EDTA to the mobile phase was critical in maintaining a stable and noise-free baseline while monitoring the signal at 10 nA. The use of disodium EDTA may have damped the effects of trace metal contaminants which were being oxidized resulting in a higher background signal.

The method described is a precise, accurate and robust method for the analysis of a new 8-aminoquinoline antimalarial agent.

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