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RAPID AND SENSITIVE QUANTITATIVE ANALYSIS OF THE NEW ANTIMALARIAL N^4-[2,6-DIMETHOXY-4-METHYL-5-[(3-TRIFLUOROMETHYL)PHENOXY]-8-QUINOLINYL]-1,4-PENTANEDIAMINE IN PLASMA BY LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

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SUMMARY

A rapid, sensitive and simple method was developed for the quantitation of the plasma concentration of N^4-[2,6-dimethoxy-4-methyl-5-[(3-trifluoromethyl)phenoxy]-8-quinolinyl]-1,4-pentanediamine, a new antimalarial active against *Plasmodium vivax*. N^4-(5-Hexoxy-6-methoxy-4-methyl-8-quinolinyl)-1,4-pentanediamine diphosphate, a similar 8-aminoquinoline, was used as an internal standard. The method involves sample clean-up by a prepacked cyano solid-phase column followed by reversed-phase liquid chromatography and oxidative electrochemical detection at +0.95 V. The assay has been validated to 5 ng/ml of plasma and is sensitive to 1 ng/ml of plasma. The results of a pilot study assessing the relative oral bioavailability of two different salt forms of the new antimalarial in dogs show the usefulness of the method for animal and human pharmacokinetic studies.

INTRODUCTION

Most of the estimated 100–300 million annual cases of malaria are due to *Plasmodium vivax* and *P. falciparum* infections. Although primaquine (I, Fig. 1) is the only clinical available drug for radical cure of *P. vivax* malaria, toxicity is a major problem with primaquine therapy especially for persons with a deficiency of glucose-6-phosphate dehydrogenase activity [1]. The search for a more potent and less toxic substitute for primaquine has yielded the primaquine derivative N^4-[2,6-dimethoxy-4-methyl-5-[(3-trifluoromethyl)phenoxy]-8-quinolinyl]-1,4-pentanediamine succinate (WR 238,605 succinate, II succinate, Fig. 1). The 8-aminoquinoline derivative II has been chosen by the U.S. Army Drug Development Program to be developed as a candidate to replace primaquine on the basis of preclinical screening tests and preliminary toxicological studies. Initial

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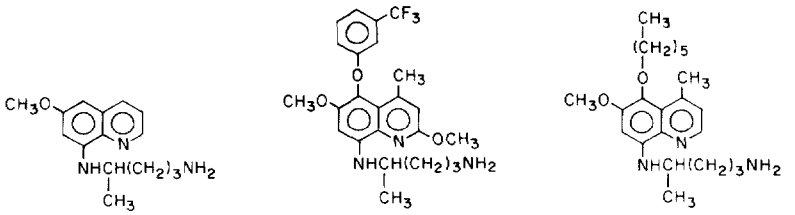

I II III

Fig. 1. Chemical structures of primaquine (I), the free base of II, and the free base of the internal standard III.

studies using the *P. cynomolgi* rhesus monkey test system [2] indicated that II succinate is 12.8 times more potent on a molar basis than primaquine in curing the rhesus monkey of *P. cynomolgi*, the simian equivalent of *P. vivax* [3]. Using a test system designed to measure methemoglobin formation in the beagle dog [4], an equal molar oral dose of II succinate caused less methemoglobin formation than the other candidate 8-aminoquinolines [5].

In order to support the development of II as a candidate antimalarial, a rapid, accurate and precise assay of the plasma concentration of unchanged II was necessary for the estimation of pharmacokinetic parameters in animals and humans. The assay described in this report has been used in a pilot study to examine the relative oral bioavailability of different salts of II.

EXPERIMENTAL

Chemicals

II succinate and N^4-(5-hexoxy-6-methoxy-4-methyl-8-quinolinyl)-1,4-pentanediamine diphosphate (WR 242,511 diphosphate, III diphosphate, Fig. 1) were synthesized on contract by Starks Assoc. (Buffalo, NY, U.S.A.) and II dihydrochloride was synthesized on contract by Ash Stevens (Detroit, MI, U.S.A.). Mefloquine, chloroquine, primaquine, quinine, halofantrine and proguanil were obtained from Walter Reed stock. Pyrimethamine was obtained from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.). HPLC-grade solvents were purchased from Burdick & Jackson Labs. (Muskegon, Mi, U.S.A.). Other chemicals were of the best commercially available grade.

Apparatus

The equipment used included: a Sorvall RT6000B centrifuge (Dupont, Newton, CT, U.S.A.); an Eppendorf microcentrifuge 5413 (Brinkmann Instruments, Westbury, NY, U.S.A.); an LC-4B electrochemical detector equipped with a glassy carbon electrode and an Ag/AgCl reference electrode (BAS, West Lafayette, IN, U.S.A.); a Gilson high-performance liquid chromatograph which included an Apple IIe controller, a Model 620 data master, a Model 303 pump equipped with a 10-ml pump head, a Model 802B pressure monitor and a Model 811 dynamic mixer (Madison, WI, U.S.A.); a WISP 710B autosampler, an RCM module and

a Nova-Pak C_{18} Radial-Pak cartridge, 10 cm × 8 mm, particle size 4 μm (Millipore-Waters, Milford, MA, U.S.A.); a Kipp & Zonen pen recorder (Delft, The Netherlands).

Standard curve representing 5–100 ng/ml II

All of the following concentrations represent the free base concentration. Human plasma (1 ml) was placed in each of eight 1.5-ml Eppendorf microcentrifuge tubes. Each plasma sample was spiked with 60 ng of the internal standard, III diphosphate (60 μl of a 1 ng/μl acetonitrile solution), directly into the plasma using a 100-μl syringe. Each sample was then spiked with a 1 ng/μl acetonitrile solution of II succinate with the following volumes: 100, 90, 70, 50, 40, 20, 10 and 5 μl, respectively. The solution of II succinate was injected directly into the plasma with either a 10- or 100-μl syringe. A 200-μl aliquot of acetonitrile was added to each plasma sample, and the plasma samples were immediately vortexed and centrifuged in the Eppendorf microcentrifuge for 2 min.

Eight cyano (CN) disposable columns (1-ml, J.T. Baker, Phillipsburg, NJ, U.S.A.) were prewashed with 1 ml of acetonitrile and 1 ml of water by vacuum. The plasma sample was added to each column being careful not to include the pellet. Each of the following steps was performed with centrifugation at 3400 *g* for 3 min. The plasma was centrifuged through the column, and each column was washed with 1 ml of water, followed by 1 ml of methanol. The wash from each step was discarded. The drugs were eluted from the columns with the final wash of 400 μl of HPLC mobile phase. The sample was vortexed and pipetted into WISP inserts.

Standard curve representing 50–1000 ng/ml II

The procedure was identical to the procedure used for the 5–100 ng/ml samples with the following exceptions. Each plasma sample was spiked with 500 ng of the internal standard, III diphosphate (50 μl of a 10 ng/μl acetonitrile solution), and with a 10 ng/μl acetonitrile solution of II succinate with the following volumes: 100, 90, 70, 40, 20, 10, 7 and 5 μl, respectively. The 3-ml cyano (CN) disposable columns were used which were washed with 3 ml of solvent at each step using vacuum. The drugs were eluted from the columns with a final wash of 2 ml of HPLC mobile phase. The sample was vortexed and pipetted into WISP vials.

Liquid chromatographic procedure

The samples were chromatographed using a high-performance liquid chromatograph equipped with a 10 cm × 8 mm Nova-Pak C_{18} Radial-Pak cartridge (Millipore-Waters). The drug and internal standard were detected with an electrochemical detector in the oxidative mode using a glassy carbon electrode and an applied voltage of 0.95 V (Ag/AgCl reference electrode). The samples were eluted at 4 ml/min with a mobile phase containing 300 ml of water, 9 ml of 85% phosphoric acid, 6.8 g of sodium acetate and 700 ml of methanol. The signal was monitored at 2 nA full scale, and 100–200 μl of each sample were injected. A sample was automatically injected every 11 min. This timing was used to avoid a late-eluting peak caused by plasma impurities coeluting with the peaks of interest

in the next sample. The late-eluting peak was chromatographically stable and caused no problem with autoinjection over extended periods.

Quantification

For each set of ten to seventeen samples, an eight-point standard curve was prepared by spiking outdated human plasma (Walter Reed Army Medical Center Blood Bank, Washington, DC, U.S.A.); anticoagulant, citrate–phosphate–dextrose–adenine) or freshly frozen (−70°C) dog plasma (Walter Reed Army Institute of Research Veterinary Facilities; anticoagulant, ethylenediaminetetraacetic acid). The peak-height ratio of II to the internal standard III versus the amount (ng) of II in the sample was fit to a calibration curve using least-squares regression. A typical standard curve in the 5–100 ng range for II in plasma is defined by the equation $y=0.013+0.041x$ with $r^2=0.997$ and a coefficient of variation equal to 4.11%. A typical standard curve in the 50–1000 ng range for II in plasma is defined by the equation $y=0.002+0.023x$ with $r^2=0.999$ and a coefficient of variation equal to 2.19%.

Additional spiked samples were treated as unknowns to evaluate the accuracy and precision of the method.

Dog subjects and drug administration

Two healthy female beagle dogs (31 months old) were supplied by the Division of Veterinary Medicine, Walter Reed Army Institute of Research, following approval of the submitted protocol. The animal care facilities and the animal care program meets the standard set forth by the "Guide for the Care and Use of Laboratory Animals", Department of Health and Human Services Publication, NIH 85-23. Each dog received tap water and food (Purina chow) ad libitum except food was withheld for 12 h prior to dosing and 4 h post-dosing. Each dog was weighed prior to dosing, and bulk drugs were placed into a gelatin capsule (24 mm × 8 mm, Size 00, Parke Davis) at a dose of 4 mg/kg (free base concentration). Both the succinate salt of II and the dihydrochloride salt of II were in powder form. During the first dosing period, one dog was given a single oral dose of the succinate salt of II and the other dog was given a single oral dose of the dihydrochloride salt of II. Five weeks after the first dose was administered, each dog received a single oral dose of the other salt form of II. Blood was drawn from the cephalic vein using a 20-gauge 3.8-cm Vacutainer needle with a 7-ml Vacutainer tube containing 0.07 ml of 15% potassium ethylenediaminetetraacetate. For each dosing period, blood was drawn pre-dose and approximately at the following times: 1, 2, 3, 4, 5, 7, 9, 11, 13, 15, 24, 48, 96, 168, 264 and 360 h after dosing. Immediately after sampling, the blood samples were centrifuged at 5°C at 1300 *g* for 5 min, and the plasma was frozen at −70°C until analyzed.

RESULTS

Retention times

Typical chromatograms of blank human plasma, spiked human plasma and dog plasma following oral administration of the succinate salt of II are shown in Fig.

2 and 3. The internal standard III elutes at 7.5 min and the drug II at 9.5 min. The apparent shorter retention times for II and III in Fig. 3 were due to a higher pump speed (see legend to Fig. 3). The blank plasma chromatogram illustrates the lack of interfering peaks in the vicinity of II and the internal standard. Although the blank plasma sample demonstrates a late-eluting plasma impurity, injecting the samples every 11 min avoided interference of the late-eluting impurity peak with the peaks of interest.

The HPLC retention times of other antimalarial compounds with which II may be administered are listed in Table I. These compounds were dissolved in mobile phase and were chromatographed using the same conditions that were used for the chromatography of II. Under these conditions, none of the antimalarials coeluted with either II or III.

Extraction efficiency

The extraction efficiency of the drug II and the internal standard III from human plasma was examined by comparing the peak heights of both drugs obtained in the chromatogram to the peak heights obtained when pure drugs were injected at concentrations expected for extraction with 100% efficiency. In the range 5–100 ng/ml II in plasma, the extraction efficiency (mean $\pm$S.D., $n=15$) was $79.7 \pm 14.9\%$ for II and $95.9 \pm 12.7\%$ for the internal standard III (60 ng/ml of plasma). No significant difference was observed in the extraction efficiency of 5 versus 100 ng of II. In the range 50–1000 ng/ml II in plasma, the extraction efficiency (mean $\pm$S.D., $n=20$) was $100.6 \pm 8.8\%$ for II and $86.1 \pm 9.8\%$ for the internal standard III (500 ng/ml of plasma). No significant difference was observed in the extraction efficiency of 50 versus 1000 ng of II in either human or dog plasma.

Method validation

Data defining the precision and accuracy of the analysis of II in the range 50–1000 ng/ml of human plasma are given in Table II. Human plasma samples spiked with 900, 200, 70 and 60 ng/ml II were analyzed as unknowns. The measurements resulted in concentrations (mean $\pm$S.D.) of 906 ± 18 ng/ml ($n=15$), 202 ± 14 ng/ml ($n=15$), 73.0 ± 5.3 ng/ml ($n=10$) and 58.6 ± 3.3 ng/ml ($n=5$), respectively. The coefficients of variation of the replicate samples were 2.0, 6.9, 7.3 and 5.6%, respectively.

Data defining the precision and accuracy of the analysis of III in the range 5–100 ng/ml of human plasma are given in Table III. Human plasma samples spiked with 90, 40, 10 and 5 ng/ml II were analyzed as unknowns. The measurements resulted in concentrations (mean $\pm$S.D., $n=15$) of 89.1 ± 3.4, 40.0 ± 1.6, 9.97 ± 0.74 and 5.04 ± 0.49 ng/ml, respectively. The coefficients of variation of the replicate samples were 3.8, 4.0, 7.4 and 9.7%, respectively.

Relative oral bioavailability of different salt forms of II

Since the anticipated route of administration of II is oral, a pilot study was performed to provide information on which salt form of II gives the highest plasma

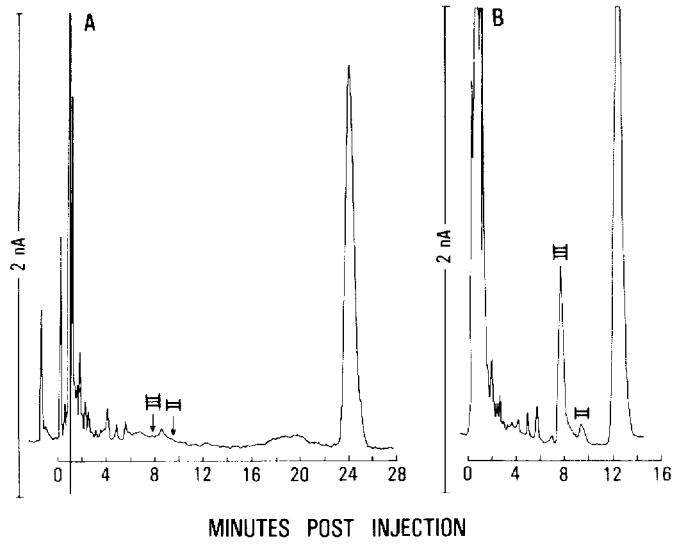

Fig. 2. Chromatograms of human plasma extracted with the 1-ml cyano columns and analyzed as described in the Experimental section. (A) Blank plasma sample (1 ml); the arrows designate the retention times for II and the internal standard III; a large peak from plasma impurity occurs at 24 min. (B) Plasma sample (1 ml) spiked with 8 ng of II succinate (free base concentration) and 60 ng of the internal standard III. Both chromatograms represent 50% of the sample.

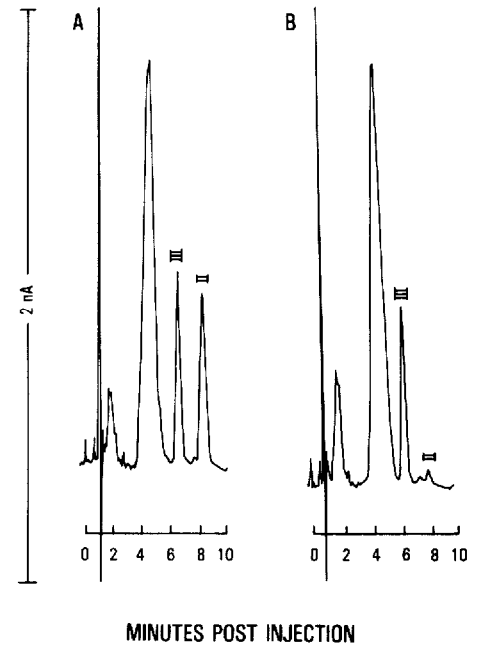

Fig. 3. Chromatograms of dog plasma 9 h (A) and 264 h (B) after receiving an oral dose of II succinate. The 1-ml samples were spiked with 500 ng of the internal standard III and extracted using the 3-ml cyano columns. Both chromatograms represent 5% of the sample and were eluted at 4.2 ml/min. The large peak at 4.5 min represents a plasma impurity from the previously injected samples.

TABLE I

HPLC RETENTION TIMES OF OTHER ANTIMALARIALS

Each of these antimalarials was chromatographed using the same conditions that were used for the analysis of compound II.

Antimalarial	Retention time (min)
Mefloquine	0.8*
Quinine	< 0.5*
Chloroquine	< 0.5
Primaquine (I)	< 0.5
Halofantrine	11.2
Pyrimethamine	< 0.5*
Proguanil	< 0.5

*Not observed with electrochemical detection under the conditions used. Retention times were obtained from peaks observed with UV detection at 254 nm.

concentration of II and to assess the appropriateness of the analytical method for pharmacokinetic studies.

Plasma concentrations of II following oral administration of either the succinate salt or the dihydrochloride salt to two beagle dogs (as described in the Experimental section) are illustrated in Fig. 4. In both dogs the peak plasma concentration of II was 25% higher after administration of the dihydrochloride salt than after administration of the succinate salt. The peak plasma concentration of II was 1.9-fold higher in dog No. 6A06 than in dog No. 6A05 for both salt forms. The peak plasma concentration occurred 7.0–12.7 h post-dosing. The area under the curve was approximately 60% greater for the dihydrochloride salt in

TABLE II

WITHIN- AND BETWEEN-DAY PRECISION AND ACCURACY DATA USING THE 50–1000 ng/ml STANDARD CURVE

The assay was validated by replicate analyses ($n=5$) of human plasma spiked with II as described in the Experimental section. C.V. = coefficient of variation.

Theoretical concentration (ng/ml)	Day No.	Analytical concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Mean C ratio*	Bias**	Within-day C.V. (%)	Between-day C.V. (%)
900	1	908 ± 5	0.6	100.9	+ 8	1.9	5.4
	2	899 ±22	2.4	99.9	− 1		
	3	912 ±24	2.6	101.3	+12		
200	1	208 ±10	4.8	104.0	+ 8	5.0	9.9
	2	210 ± 7	3.3	105.0	+10		
	3	188 ±13	6.9	94.0	−12		
70	1	74.7± 1.8	2.4	106.7	+ 4.7	6.3	1.8
	2	71.2± 7.2	10.1	101.7	− 1.2		
60	1	58.6± 3.3	5.6	97.7	− 1.4		

*Ratio of mean analytical concentration to theoretical concentration × 100.

**Mean analytical concentration − theoretical concentration.

TABLE III

WITHIN- AND BETWEEN-DAY PRECISION AND ACCURACY DATA USING THE 5-100 ng/ml STANDARD CURVE

The assay was validated by replicate analyses ($n=5$) of human plasma spiked with II as described in the Experimental section. The replicate analyses were repeated on each day of three days. C.V. = coefficient of variation.

Theoretical concentration (ng/ml)	Day No.	Analytical concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Mean *C* ratio*	Bias**	Within-day C.V. (%)	Between-day C.V. (%)
90	1	87.9 ±3.6	4.1	97.7	−2.1	3.2	2.6
	2	87.7 ±2.5	2.9	97.4	−2.3		
	3	91.8 ±2.3	2.5	102.0	+1.8		
40	1	39.3 ±1.3	3.3	98.3	−0.7	3.9	1.8
	2	40.7 ±1.9	4.7	101.8	+0.7		
	3	40.0 ±1.5	3.8	100.0	0.0		
10	1	9.79 ± 0.77	7.9	97.9	−0.21	7.2	19.3
	2	10.24 ± 0.56	5.5	102.4	+0.24		
	3	9.89 ± 0.80	8.1	98.9	−0.11		
5	1	4.91 ± 0.22	4.5	98.2	−0.09	8.7	16.8
	2	4.94 ± 0.52	10.5	98.8	−0.06		
	3	5.28 ± 0.58	11.0	105.6	+0.28		

*Ratio of mean analytical concentration to theoretical concentration × 100.

**Mean analytical concentration − theoretical concentration.

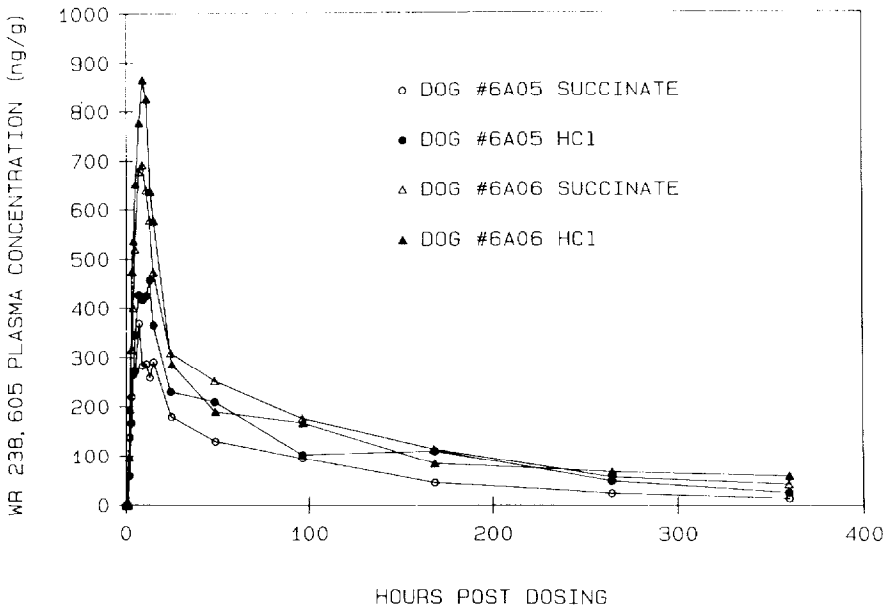

Fig. 4. Composite graph of the plasma concentration of II following a single oral dose of either the dihydrochloride or the succinate salt (4 mg/kg, free base concentration) to beagle dogs. The plasma samples were collected and analyzed as described in the Experimental section.

dog No. 6A05, but was essentially equal for both salts in dog No. 6A06. Compound II was not detectable in any of the pre-dose plasma samples indicating that the time between doses was sufficient to prevent measurable residual drug concentration at the time of the second dose.

In comparison to I, II was eliminated much more slowly. A study examining the oral pharmacokinetics of a single oral dose of 1 mg/kg I as its diphosphate salt in six beagle dogs resulted in a peak plasma concentration of I at 1–2.5 h post-dosing and a plasma elimination half-life of 2.05 ± 0.46 h [6].

DISCUSSION

The method described in this report represents a precise and accurate analysis of the new 8-aminoquinoline antimalarial II in plasma. The method is sensitive to 1 ng/ml (as defined by a signal-to-noise ratio of 4). The use of prepacked extraction columns made sample clean-up simpler and more rapid than more cumbersome liquid–liquid extraction methods. An important feature of the extraction procedure was the preparation of the spiking solutions of II and the internal standard III in acetonitrile and the addition of the acetonitrile to the plasma prior to the extraction. In the absence of acetonitrile, the drugs coeluted through the cyano columns with the plasma. The preparation of the drug and internal standard spiking solutions in methanol also caused the drugs to coelute with the plasma.

Electrochemical detection was chosen over UV detection for two reasons. First, detection of II by oxidative electrochemistry at 2 nA full scale and 0.95 V was six-fold more sensitive than UV detection at 254 nm and 0.005 a.u.f.s. Second, there were substantially fewer interfering peaks with electrochemical detection. Although the method described in this report was developed for the analysis of II, it should be equally useful for the quantitation of the internal standard III, another potential candidate 8-aminoquinoline antimalarial for the treatment of *P. vivax* [4].

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