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Quantification of the individual enantiomer plasma concentrations of the candidate antimalarial agent N^4-[2,6-dimethoxy-4-methyl-5-[(3-trifluoromethyl)phenoxy]-8-quinolinyl]-1,4-pentanediamine (WR 238,605)

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Abstract

A high-performance liquid chromatographic method was developed to quantitate the plasma concentrations of the individual enantiomers of a candidate 8-aminoquinoline antimalarial agent WR 238,605 (I). The method employed one-step liquid extraction of a 0.5-ml plasma sample followed by direct injection of the extract through a chiral column and detection by fluorescence. Quantification was achieved using an internal standard. The limit of quantification was 10 ng/ml for each enantiomer. The method is sufficiently sensitive to quantitate the plasma concentrations of both enantiomers for 30 days following a single oral dose of 400 mg of the antimalarial agent administered as the racemic succinate salt to healthy human male volunteers. In nearly all samples taken 12 h to 30 days post-dose from three subjects, the difference in the plasma concentrations of the two enantiomers is less than 10%.

1. Introduction

Racemic N^4-[2,6-dimethoxy-4-methyl-5-[(3-trifluoromethyl)phenoxy]-8-quinolinyl]-1,4-pentanediamine (WR 238,605, I, Fig. 1) as its succinate salt is a candidate 8-aminoquinoline antimalarial agent presently undergoing phase I and early phase II clinical studies. In vivo studies in mice [1] and monkeys [2] have shown compound I to be more efficacious and less toxic than primaquine, the primary agent presently available for radical cure of *Plasmodium vivax* and *P. ovale* infections. Compound I is effective against a variety of stages in the life cycle of the parasite including the pre-erythrocytic stage, the drug-sensitive and drug-resistant asexual intra-erythrocytic stage, gametocytes, and for *P. cynomolgi* the intra-hepatic hypnozoite stage [1,2]. Compound I is, therefore, a promising radical curative agent against human *P. vivax* infections as well as a promising causal prophylactic agent against all human *Plasmodium* infections including multi-drug resistant infections.

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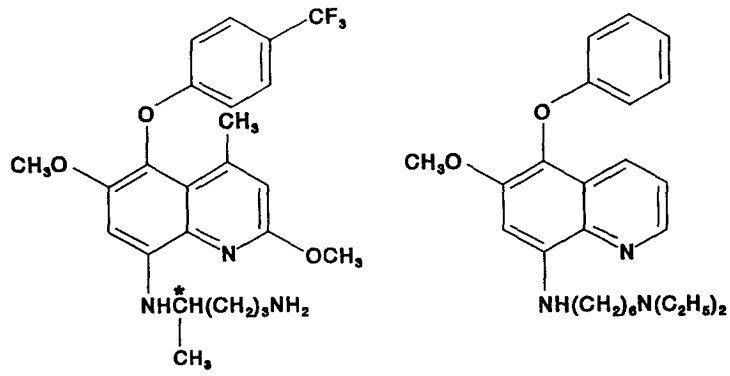

I II

Fig. 1. Structures of WR 238,605 (I) and the internal standard (II). The chiral carbon atom of I is denoted by an asterisk.

The present assay was developed in order to determine the relative bioavailability and the relative plasma elimination rate of the individual enantiomers of I.

2. Experimental

2.1. Chemicals

Compound I as its succinate salt was synthesized on contract by Starks Assoc. (Buffalo, NY, USA) and has a purity of 99.9(±0.5)% based upon spectral, chromatographic, and elemental data [3]. The internal standard, N^1-diethyl-N^6-[6-methoxy-5-phenoxy-8-quinolinyl]-1,6-hexanediamine (WR 211,819, II) (Fig. 1), as the hydrochloride salt and the metabolite of I, 8-amino - 2,6 - dimethoxy - 4 - methyl - 5 - [(3 - trifluoromethyl)phenoxy]quinoline as its methylsulfonate salt, were obtained from the Walter Reed Army Institute of Research Repository (Washington, DC, USA). Hexane, 2-propanol, and diethylamine were obtained commercially and were of HPLC grade. Outdated human plasma was obtained from the Walter Reed Army Medical Center Blood Bank.

2.2. Apparatus

All plasma samples were stored at −80°C 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 (Brinkmann Instruments, Westbury, NY, USA). The HPLC system consisted of a refrigerated Waters WISP 710B autosampler (Milford, MA, USA), a Gilson 202 pump (Middleton, WI, USA), a Daicel Chiralpak AD 25 × 0.46 cm column (J.T. Baker, Phillipsburg, NJ, USA), an Eppendorf CH-30 column heater, a Shimadzu RF-535 fluorescence detector (Columbia, MD, USA), and an Axxiom controller/integrator (Calabasas, CA, USA).

2.3. Standard curve and validation samples

Standard curves were run for each validation series and each subject. Stock solutions of I (5 ng/μl and 20 ng/μl) and II (100 ng/μl) in 2-propanol were stored in the dark at 4°C. The eight plasma samples used for the standard curve were prepared by spiking 0.5 ml of plasma with 1, 2, 4, or 8 μl of the 5 ng/μl solution of I or 4, 6, 8, or 10 μl of the 20 ng/μl solution of I. Each sample was then spiked with 10 μl of solution II. The plasma samples were extracted with a mixture of 2-propanol–hexane (3:5) containing 0.4% diethylamine (0.4 ml) by vortex-mixing for 10 s and centrifuging at 8800 *g* and ambient temperature for 3 min. The organic layer was pipetted off and placed into micro WISP vials. Validation

samples were prepared in exactly the same manner as the standard curve samples.

2.4. Extraction efficiency

Extraction efficiency was determined by extracting both unspiked 0.5-ml plasma samples and 0.5-ml plasma samples spiked with 1000 ng of II and 10 or 200 ng of I. All plasma samples were extracted as described above. Following transfer of the organic layer to micro WISP vials, the extract of the unspiked plasma samples was spiked with 1000 ng of II and 10 or 200 ng of I. The extraction efficiency was defined as the ratio of the peak height of the enantiomers of I and the internal standard to the peak height of the corresponding peaks in the chromatograms of the samples spiked after plasma extraction.

2.5. Liquid chromatographic and detection procedures

The standard curve WISP vials and sample-containing WISP vials were refrigerated in the WISP at 0°C. The mobile phase consisted of 2-propanol–hexane (70:30, v/v) with 0.4% diethylamine pumped at 1 ml/min. The column heater was set to 30°C. Each sample run lasted 8 min. The detector was set to high sensitivity, 270 nm for the excitation wavelength, and 470 nm for the emission wavelength. A 50-μl aliquot was injected.

2.6. Quantification

The concentration of each enantiomer of I in extracted experimental samples was determined by comparison of the assayed peak-height ratios (drug to IS) to those of a standard curve generated with each run. Standard curves were derived by non-weighted least-squares linear regression and fit to the equation $y = mx + b$, where m equals the slope and b equals the intercept.

2.7. Clinical study

Healthy male volunteers, ages 18–32, were given a single oral dose of 400 mg (free base quantity) of racemic compound I as the succinate salt [4]. Blood was drawn pre-dose and at specified times following dose administration. Blood samples were centrifuged immediately following collection, and the plasma fraction was frozen at −80°C until analysis. Each 0.5-ml sample was spiked with 10 μl of the 100 ng/μl solution of II in 2-propanol and extracted as described above for the standard curve samples.

2.8. Chromatography of metabolite

Compound I and its metabolite were chromatographed on a Waters Symmetry C_{18} column, 3.9 × 150 mm, eluted with a mixture of acetonitrile–water–diethylamine (80:20:0.4, v/v) at 1 ml/min. The metabolite and compound I eluted at 4.2 and 5.5 min, respectively. Detection was by fluorescence as described above.

3. Results

3.1. Retention times

Typical chromatograms of blank human plasma, spiked human plasma, and human plasma following a single oral dose of I are shown in Figs. 2 and 3. The enantiomers of I elute at 4.1 min (peak 1) and 6.9 min (peak 2). The internal standard elutes at 5.5 min.

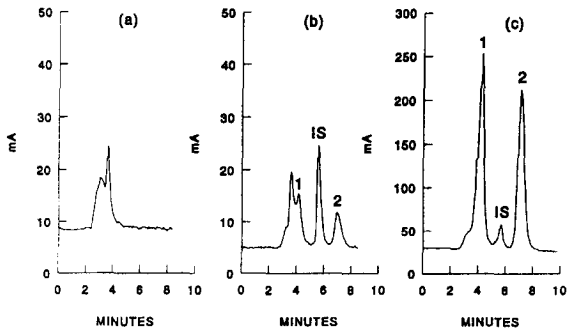

Fig. 2. Chromatograms of extracts of (a) 0.5 ml of blank human plasma, (b) 0.5 ml of human plasma spiked with 10 ng/ml of each enantiomer of I and 200 ng/ml of II, and (c) 0.5 ml of human plasma spiked with 200 ng/ml of each enantiomer of I and 200 ng/ml of II.

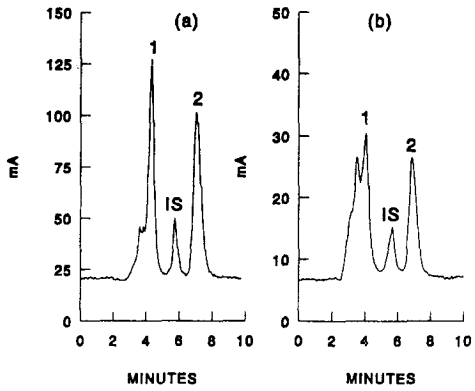

Fig. 3. Chromatograms of extracts of 0.5-ml plasma samples from a human volunteer (a) 24 h post-dosing (93.9 ng/ml of peak 1 and 104.1 ng/ml of peak 2 were detected) and (b) 30 days post-dosing (34.0 ng/ml of peak 1 and 34.9 ng/ml of peak 2 were detected).

The blank human plasma chromatogram demonstrates the lack of interfering peaks in the vicinity of the internal standard and peak 2. There is a small interfering peak in the human plasma samples adjoining peak 1. This interfering peak could be separated from peak 1 by reducing the concentration of 2-propanol in the mobile phase or cooling the HPLC column. However, these actions caused the internal standard peak and peak 2 to unacceptably broaden. Thus, we chose to retain the analysis conditions presented in this report since the interference to peak 1 only affects the quantification at low ng/ml concentrations, concentrations lower than observed in the clinical samples. If removal of the interfering plasma contaminant is desired, column switching of the peak for compound I from the achiral HPLC column described for the metabolite chromatography in the Section 2 to the chiral column should be workable.

3.2. Extraction efficiency

When plasma samples ($n = 3$) were spiked with 200 ng/ml of I and 1000 ng/ml of II, the extraction efficiency of peak 1 was 84.9(±4.1)%, of peak 2 was 86.3(±8.8)%, and of II was 82.7(±10.2)%. When plasma samples ($n = 3$) were spiked with 10 ng/ml of I and 1000 ng/ml of II, the extraction efficiency of peak 1 was 85.7(±5.4)%, of peak 2 was 88.3(±9.6)%, and of II was 79.3(±3.0)%. The numbers in parentheses represent standard deviations.

3.3. Quantification

A typical standard curve from 5 to 200 ng/ml ($n = 8$) for the peak 1 isomer is represented by the equation $y = 0.0364(\pm 0.0005)x + 0.1960$ (± 0.0539), $r^2 = 0.9988$, where the numbers in parentheses are the standard errors. A representative curve from 5 to 200 ng/ml ($n = 8$) for the peak 2 isomer is described by the equation $y = 0.0309(\pm 0.0004)x + 0.0117(\pm 0.0384)$, $r^2 = 0.9992$.

3.4. Method validation

Data defining the precision and accuracy of the quantification of the enantiomers of I in the range of 5–200 ng/ml are given in Tables 1 and 2. Accuracy of the method was determined by analyzing plasma samples spiked with 5, 10, 40, 120, and 200 ng/ml of each enantiomer. Precision of the method over the 5–200 ng/ml range was determined by the analysis of replicate spiked samples freshly prepared each day for a period of 5 days.

The method's limit of quantification for each enantiomer is 10 ng/ml from a 0.5-ml sample based upon the accuracy of replicate spiked plasma samples (Tables 1 and 2). Although the precision of the determinations at 5 ng/ml was within acceptable limits for the limit of detection with the mean inter-day and intra-day R.S.D. within 15%, the quantity of peak 1 was always overestimated by at least an average of 34%. The quantity of peak 2 could not be accurately measured every day with the average measured amount being more than 20% different than the spiked amount on days 2 and 5.

3.5. Stability of enantiomers

Compound I is stable in human plasma to storage at −20°C and thawing for at least 134 days [5]. The 12 h post-dosing sample from one of the volunteers had a peak 2 to peak 1 quantity

Table 1
Precision and accuracy of the assay of peak 1

Theoretical concentration (ng/ml)	Day	n	Analytical concentration[a] (ng/ml)	R.S.D. (%)		Error[b] (absolute value) (%)
				Within-day	Between-day	
200	1	5	229.4 ± 12.9	5.6	6.4	14.7
	2	5	205.7 ± 8.6	4.2		2.8
	3	5	189.4 ± 3.6	1.9		−5.3
	4	5	204.8 ± 3.8	1.9		2.4
	5	5	210.2 ± 7.3	3.5		5.1
120	1	5	130.9 ± 1.6	1.2	3.6	9.1
	2	5	119.8 ± 5.7	4.8		−0.1
	3	5	122.4 ± 7.6	6.2		2.0
	4	5	128.4 ± 5.8	4.5		7.0
	5	5	120.9 ± 4.9	4.1		0.7
40	1	5	42.7 ± 1.0	2.3	5.4	6.7
	2	5	41.9 ± 1.3	1.3		4.6
	3	5	47.6 ± 0.9	1.9		19.0
	4	5	43.2 ± 1.1	2.5		8.0
	5	5	41.7 ± 6.1	1.5		4.3
10	1	5	10.5 ± 0.7	6.7	4.6	4.7
	2	5	11.6 ± 0.6	5.2		15.5
	3	5	11.2 ± 0.8	7.1		12.4
	4	5	11.8 ± 1.0	8.5		18.2
	5	5	11.6 ± 0.3	2.6		15.8
5	1	5	6.71 ± 0.90	13.4	11.6	34.1
	2	3	7.25 ± 0.28	3.9		44.9
	3	5	7.01 ± 0.86	12.3		40.2
	4	5	8.34 ± 0.26	3.2		66.8
	5	5	6.86 ± 0.41	6.0		37.2

[a] Mean ± S.D.
[b] Error is defined as [(measured concentration − theoretical concentration)/theoretical concentration] × 100.

ratio of 1.15 at initial analysis and a 1.14 quantity ratio when re-analyzed after 7 or 71 days of additional storage at −80°C. No observable racemization occurred when the individual isomer peaks which had been collected off the HPLC column were re-injected either immediately or after storage in methanol at 0°C in the dark for over 6 months.

3.6. Clinical study

The plasma concentrations of the enantiomers of I following a single oral dose of 400 mg of I are illustrated in Fig. 4. The data demonstrate that the candidate antimalarial agent has a long plasma elimination phase with measurable concentrations 30 days post dosing. The data also demonstrate that the plasma concentrations of the two enantiomers are nearly equal throughout the sampling period.

4. Discussion

The method described in this report represents a precise and accurate method for quantitating the enantiomers of a candidate 8-aminoquinoline antimalarial agent. The limit of accurate and precise quantification is 10 ng/ml which is sufficient to provide pharmacokinetic data with respect to the plasma concentrations of the enantiomers of I. Thirty days after a single oral dose of racemic I, the plasma concentration of each enantiomer was still greater than 20 ng/ml.

Table 2
Precision and accuracy of the assay of peak 2

Theoretical concentration (ng/ml)	Day	n	Analytical concentration[a] (ng/ml)	R.S.D. (%)		Error[b] (absolute value) (%)
				Within-day	Between-day	
200	1	5	221.4 ± 11.1	5.0	5.7	10.7
	2	5	203.6 ± 7.4	3.6		1.8
	3	5	188.4 ± 4.5	2.4		−5.8
	4	5	215.4 ± 3.7	1.7		7.7
	5	5	207.0 ± 9.2	4.4		3.5
120	1	5	128.8 ± 1.8	1.4	5.9	7.3
	2	5	117.2 ± 5.7	4.9		−2.3
	3	5	120.6 ± 7.7	6.4		0.5
	4	5	135.6 ± 6.6	4.9		13.0
	5	5	118.0 ± 5.8	4.9		−1.7
40	1	5	42.0 ± 0.8	1.9	6.0	5.1
	2	5	40.9 ± 1.1	2.7		2.4
	3	5	46.5 ± 1.1	2.4		16.2
	4	5	43.9 ± 0.7	1.6		9.7
	5	5	39.7 ± 3.0	7.5		−0.7
10	1	5	10.4 ± 0.1	0.9	3.7	4.4
	2	5	10.5 ± 0.4	3.8		5.3
	3	5	10.9 ± 0.7	6.5		9.1
	4	5	10.5 ± 0.4	3.8		5.3
	5	5	11.4 ± 0.4	3.5		14.4
5	1	5	5.35 ± 0.28	5.2	10.8	7.1
	2	3	6.27 ± 0.22	3.5		25.4
	3	5	5.87 ± 0.28	4.7		17.4
	4	5	5.42 ± 0.34	6.3		8.4
	5	5	6.78 ± 0.40	5.9		35.6

[a] Mean ± S.D.
[b] Error is defined as [(measured concentration − theoretical concentration)/theoretical concentration] × 100.

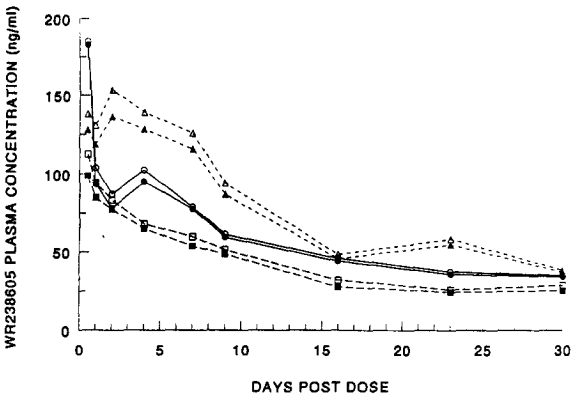

Fig. 4. Concentration–time profile for three subjects following a single oral dose of 400 mg of the succinate salt of racemic compound I. Peak 1, solid symbols. Peak 2, open symbols. Subject 1, triangles; subject 2, circles; subject 3, squares.

Thus, this assay provides individual pharmacokinetic data for the two enantiomers of I with respect to the relative rate of absorption, metabolism and/or elimination of the individual enantiomers of I.

Samples are refrigerated at 0°C while awaiting chromatography since at ambient room temperature a reduction in peak size for both enantiomers of I versus the size of the internal standard peak (II) occurs within a few hours. Although the internal standard is stable awaiting chromatography at ambient room temperature for at least 9 h, peak 1 was stable for 3 h but decomposed approximately 25% by 4 h. Although stable for at least 1 h, peak 2 was less stable than peak 1 decomposing 30% by 3 h. With refrigeration the enantiomers of I are stable for at least 30

h. The HPLC column is heated slightly above room temperature (30°C) since variation in room temperature causes retention times to shift resulting in changes in the peak-height ratio between the enantiomers of I and the internal standard (II).

Since chiral HPLC columns do not generally have the selectivity of achiral columns, there was a possibility that metabolites of I could interfere with the chromatography of the enantiomers in the clinical samples. The major primary metabolite of I obtained from incubation of I with rat liver microsomes [6] has been identified as the conversion of the 1,4-pentane diamine side chain to a primary amine group. Since this metabolite co-elutes with peak 1 on the Chiralpak AD column, the clinical plasma samples were re-examined using an achiral C_{18} column to resolve I from its metabolite. When the clinical plasma samples were extracted in the same manner as used for the quantification of the enantiomers of I and chromatographed on the C_{18} column, essentially no peaks other than the peak for compound I was observed. Thus, no interference from this potential metabolite was evident in the clinical plasma samples.

This method represents a simple, accurate, precise, and rapid method for the quantification of the enantiomers of a candidate 8-aminoquinoline antimalarial agent presently undergoing clinical study.

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