SUPPLEMENTAL MATERIALS

The supplementary material contains the following data:

1. Summary of drug analysis of amodiaquine (AQ), desethylamodiaquine (DAQ), artemisinin (ARN), artesunate (ARS), dihydroartemisinin (DHA), and piperaquine (PPQ)
2. Liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis of AQ and DAQ
3. LC/MS/MS analysis of ARN
4. LC/MS/MS analysis of ARS and DHA
5. LC/MS/MS analysis of PPQ
6. Supplemental Table 13. Plasma concentrations of PPQ and DAQ after 3-day regimens of Artequick and Coarsucam in healthy Vietnamese volunteers.

SUPPLEMENTAL MATERIAL 1

SUMMARY OF DRUG ANALYSIS OF AQ, DAQ, ARN, ARS, DHA, AND PPQ

Plasma concentrations of AQ, DAQ, ARN, ARS, DHA, and PPQ were measured on an AB Sciex 4000 QTRAP LC-MS/MS analyzer (Applied Biosystems, Concord, Ontario, Canada) preceded by a Prominence liquid chromatography system (Shimadzu, Canby, OR), including a temperature-controlled autosampler. The mobile phases were subjected to electrospray ionization in the positive ion mode, and elution of analytes was confirmed by multiple-reaction monitoring (MRM). In brief, the lower limit of quantification (LLOQ) of AQ and DAQ in plasma was 0.39 and 3.91 ng/mL ($N = 5$), respectively, with an inaccuracy of ≤ 1.79%, using 50 µL of sample. The inter-assay precision of analysis (percent coefficient of variation [CV]) for AQ and DAQ across the concentration range of 0.39–100 ng/mL and 3.91–2,000 ng/mL ($N = 7$–10), respectively, was ≤ 6.91%. The LLOQ of ARN in plasma was 1 ng/mL, with an inaccuracy of ≤ 4.44%, using 50 µL of sample. The inter-assay precision of analysis for ARN across the concentration range of 1–1,000 ng/mL was ≤ 12.26% ($N = 10$–21). The LLOQ of ARS, and DHA in plasma was 1.19 and 1.96 ng/mL, respectively, with an inaccuracy of ≤ 2.69% ($N = 5$), using 50 µL of sample. The inter-assay precision of analysis for ARS and DHA across the concentration range of 1.19–728 ng/mL ($N = 17$–28) and 1.96–2,500 ng/mL ($N = 16$–27), respectively, was ≤ 14.48%. The LLOQ of PPQ in plasma was 0.25 ng/mL, with an inaccuracy of ≤ 1.5%, using 50 µL of sample. The inter-assay precision of analysis for PPQ across the concentration range of 0.25–1,000 ng/mL was ≤ 11.9%.

SUPPLEMENTAL MATERIAL 2

LC/MS/MS ANALYSIS OF AQ AND DAQ

Standards.

- Amodiaquine: Sample no. RMAQ 20100608-03. Worldwide Antimalarial Resistance Network Reference compound.
- Desethylamodiaquine: Sample no. RMAQm 20100526-18. Worldwide Antimalarial Resistance Network Reference compound.
- Internal standard: stable isotope labeled (SIL) AQ: Sample no. RMSILAQ 20110602-04. Worldwide Antimalarial Resistance Network Reference compound.
- Internal standard: SIL DAQ: Sample no. RMSILAQm 20110602-01. Worldwide Antimalarial Resistance Network Reference compound.

Chromatographic conditions.

- Liquid chromatography column: Phenomenex Luna Pentafluorophenyl (PFP) (2) 50 mm × 2.1 mm internal diameter (I.D.) 5 µm, 00B-4448-B0.
- Pre-column: Phenomenex PFP 4 mm × 2.0 mm I.D. 5 µm, AJO-8326.
- Mobile phase flow: isocratic, 0.5 mL/minute (24% mobile phase A, 76% mobile phase B).
- Mobile phase A: 5 mM ammonium acetate–0.1% formic acid.
- Mobile phase B: acetonitrile (ACN).
- Run time: 3.5 minutes.

Mass spectrometer conditions:

Curtain gas	30
Collision activated dissociation gas	Medium
Ion spray (voltage)	3,500
Temp (°C)	500
Nebuliser gas	65
Heater gas	60
Interface heater	On

Mass spectrometry transitions:

Analyte	Q1 (Da)	Q3 (Da)	Time (ms)	DP (v)	EP (v)	CE (v)	CXP (v)
AQ	356.20	283.10	100	66	10	25	24
SIL AQ	336.25	283.10	100	76	10	27	20
DAQ	328.09	283.10	100	56	10	23	20
SIL DAQ	333.28	283.10	100	66	10	25	24

AQ = amodiaquine; CE = collision energy; CXP = collision cell exit potential; DAQ = desethylamodiaquine; DP = declustering potential; EP = entrance potential; SIL = stable isotope labeled.

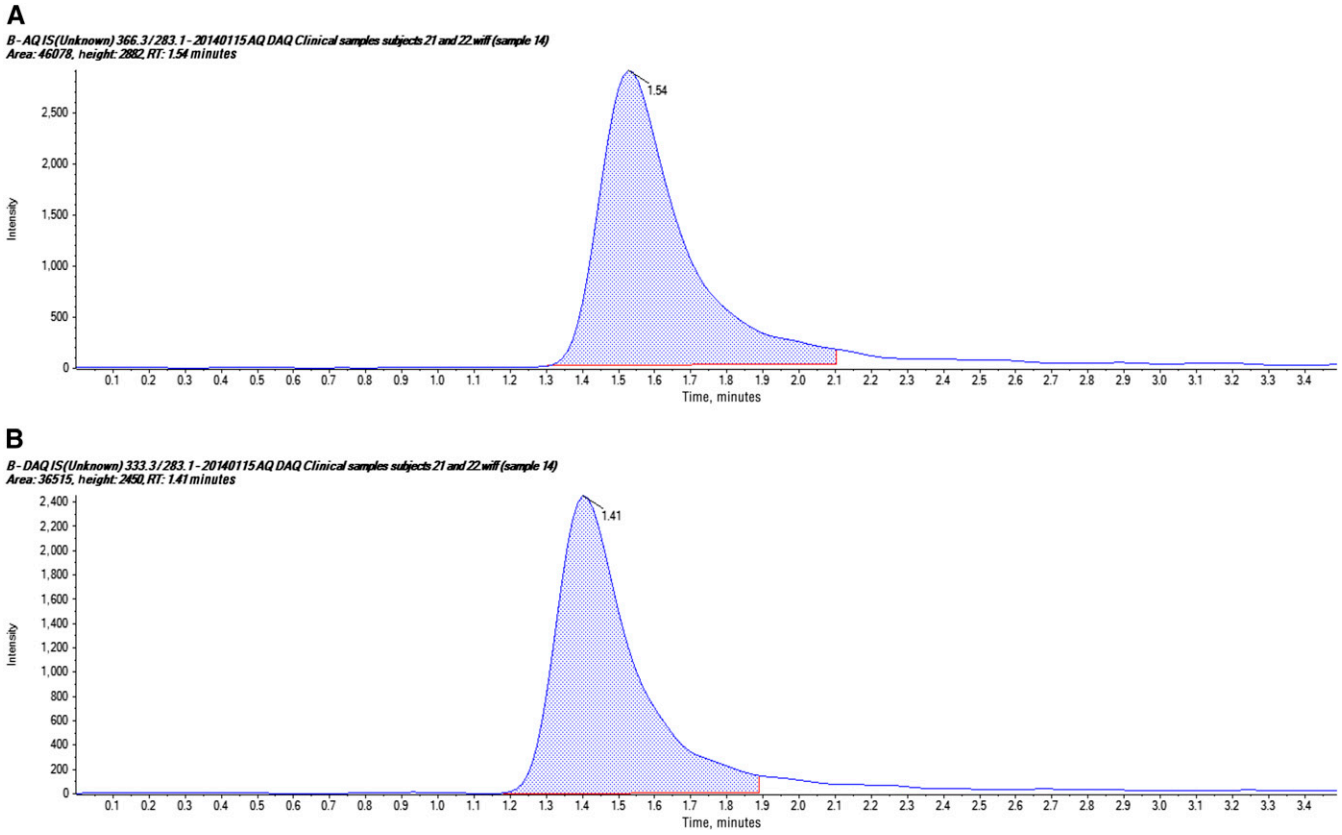
Subject plasma sample preparation.

- Subject plasma samples were thawed in a room-temperature circulating water bath and were vortex mixed before analysis.
- Calibrators and quality controls (QCs) were prepared by spiking AQ- and DAQ-free citrate phosphate dextrose anticoagulated human plasma (50 µL) with ACN containing internal standard (1 ng/mL SIL AQ and SIL DAQ in ACN; 250 µL) or blank ACN (250 µL) for the double blank.
- Hundred microliters of 3 M $ZnSO_4$ was then added to all tubes to enhance protein precipitation. Calibrators and blanks were then treated as unknowns.
- The tubes were mixed by vortexing for 3 minutes at maximum vortex setting. The tubes were then centrifuged (20,817 × *g*, 5 minutes at 4°C).
- The organic layer (100 µL) was subsequently transferred to a 96-well polypropylene microtitre plate containing 100 µL of 0.1% formic acid and 5 mM ammonium acetate.
- Two microliters was injected onto the column.

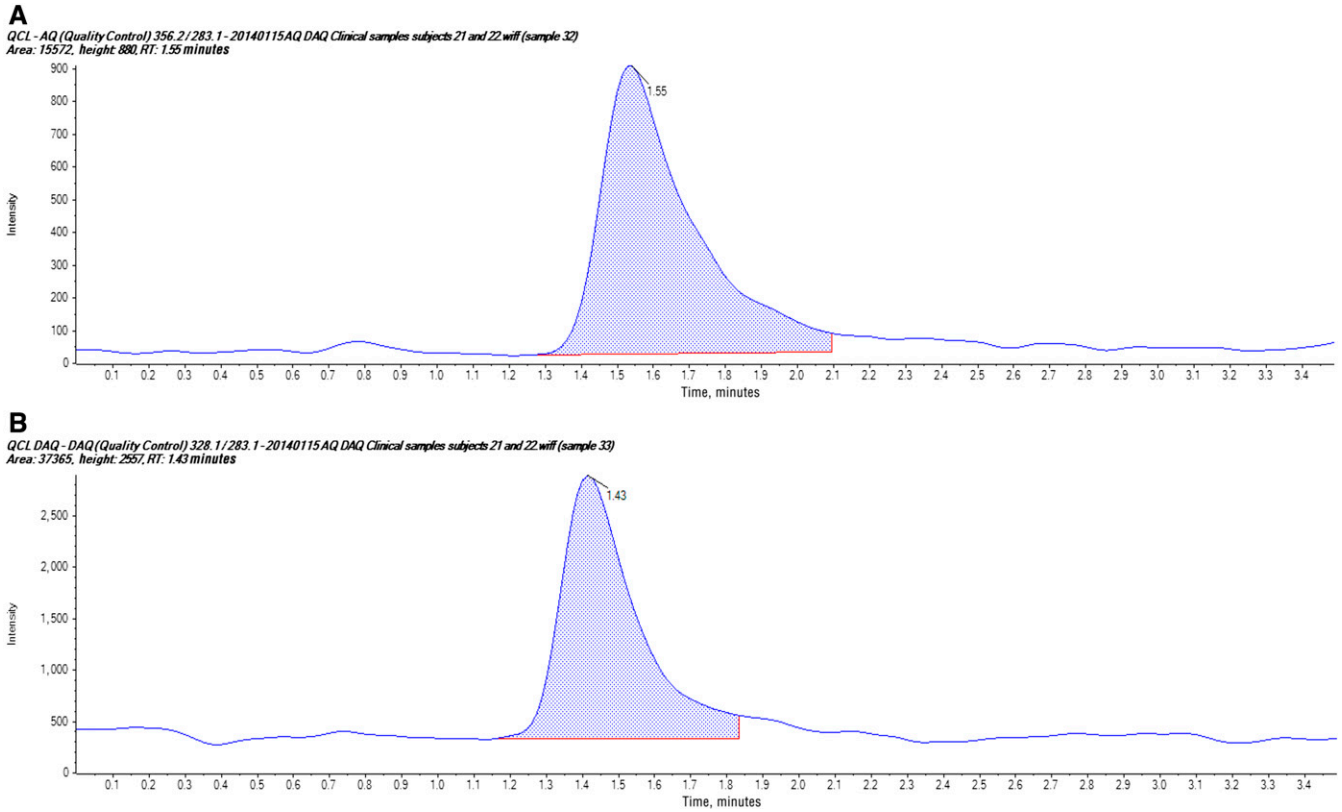
Calculation of results. Chromatograms were integrated and processed using Applied Biosystems software (Analyst V1.4.2). Concentrations of AQ and DAQ in subject plasma samples and QCs were calculated from regression curves of peak area ratios (AQ: SIL AQ and DAQ:SIL DAQ) versus analyte concentration using Multiquant 2.1 software (Concord, Ontario, Canada). A weighting of $(1/x^2)$ was used to generate a quadratic regression curve.

Testing of drug-free plasmas for ion suppression. Six drug-free plasma samples were tested for matrix effect (ion suppression) to identify a drug-free plasma that showed no identifiable effect on drug quantification. This was performed by infusing a low quantity of AQ (10 ng/mL) and DAQ (10 ng/mL) while injecting ACN extracts from the six drug-free plasmas. None of the plasmas showed ion suppression and one of these, 2HSB003/09, was used to prepare standards and QC samples.

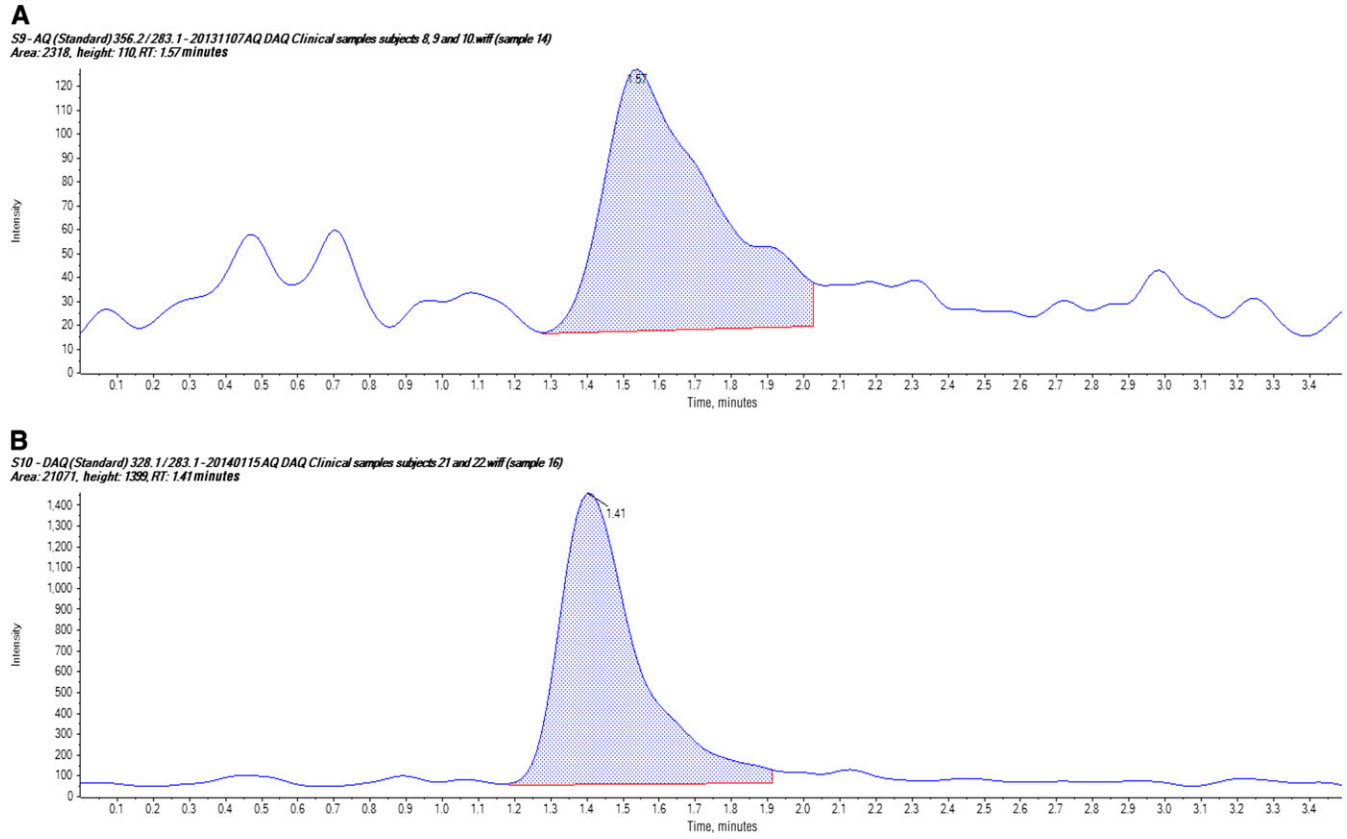
Chromatograms. Typical chromatograms of AQ, DAQ, and the internal standards SIL AQ and SIL DAQ in plasma are shown in Supplemental Figures 1–6. They are internal standards SIL AQ and SIL DAQ (Supplemental Figure 1); low-range QC plasma AQ and DAQ (Supplemental Figure 2); a low calibration standard (Supplemental Figure 3); a high calibration standard (Supplemental Figure 4); subject plasma at time = 0 hour (Supplemental Figure 5); and subject plasma at time = 1 hour (Supplemental Figure 6). The retention times for AQ, SIL AQ, DAQ, and SIL DAQ were approximately 1.55, 1.54, 1.42, and 1.41 minutes, respectively.



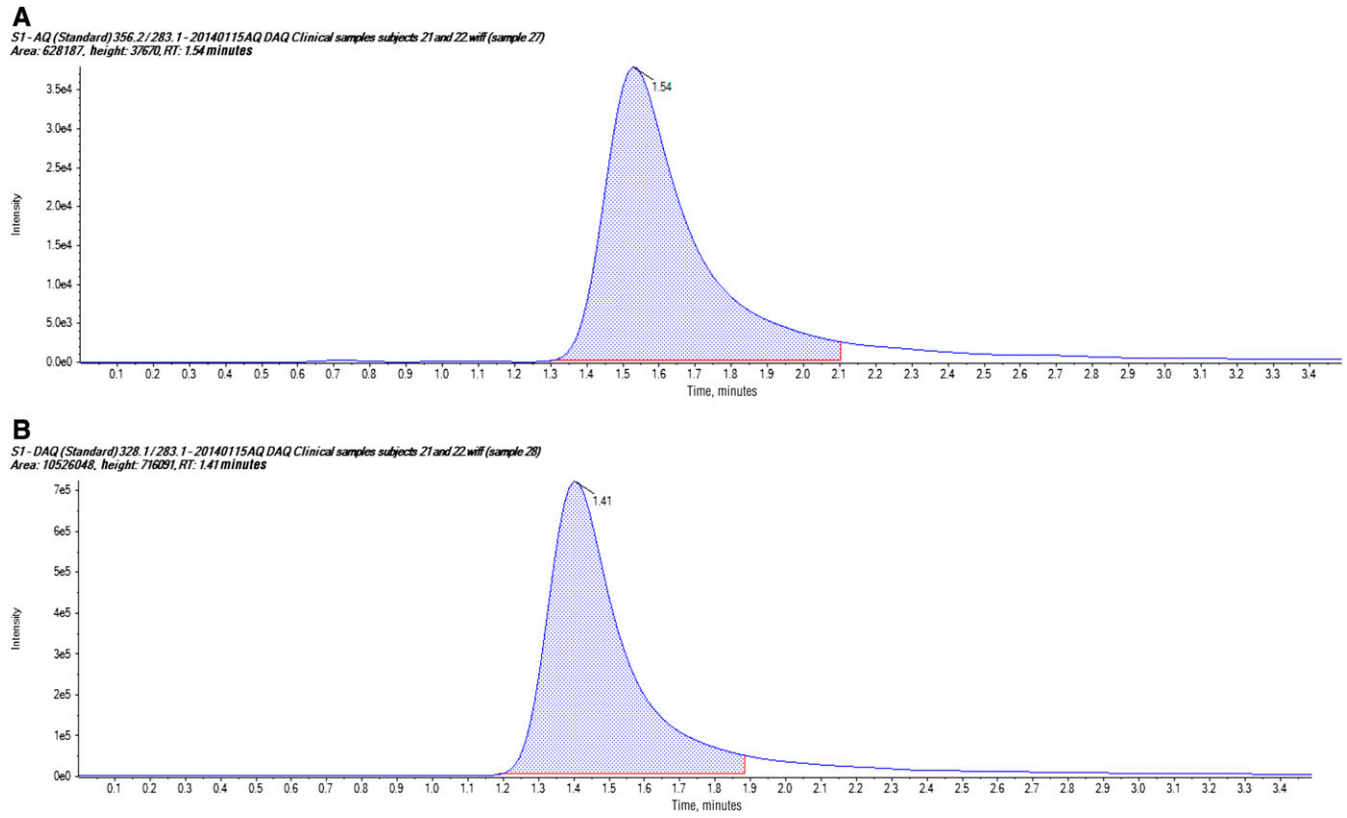
SUPPLEMENTAL FIGURE 1. Internal standards. (**A**) SIL AQ (1 ng/mL). (**B**) SIL DAQ (1 ng/mL). AQ = amodiaquine; DAQ = desethylamodiaquine; SIL = stable isotope labeled.



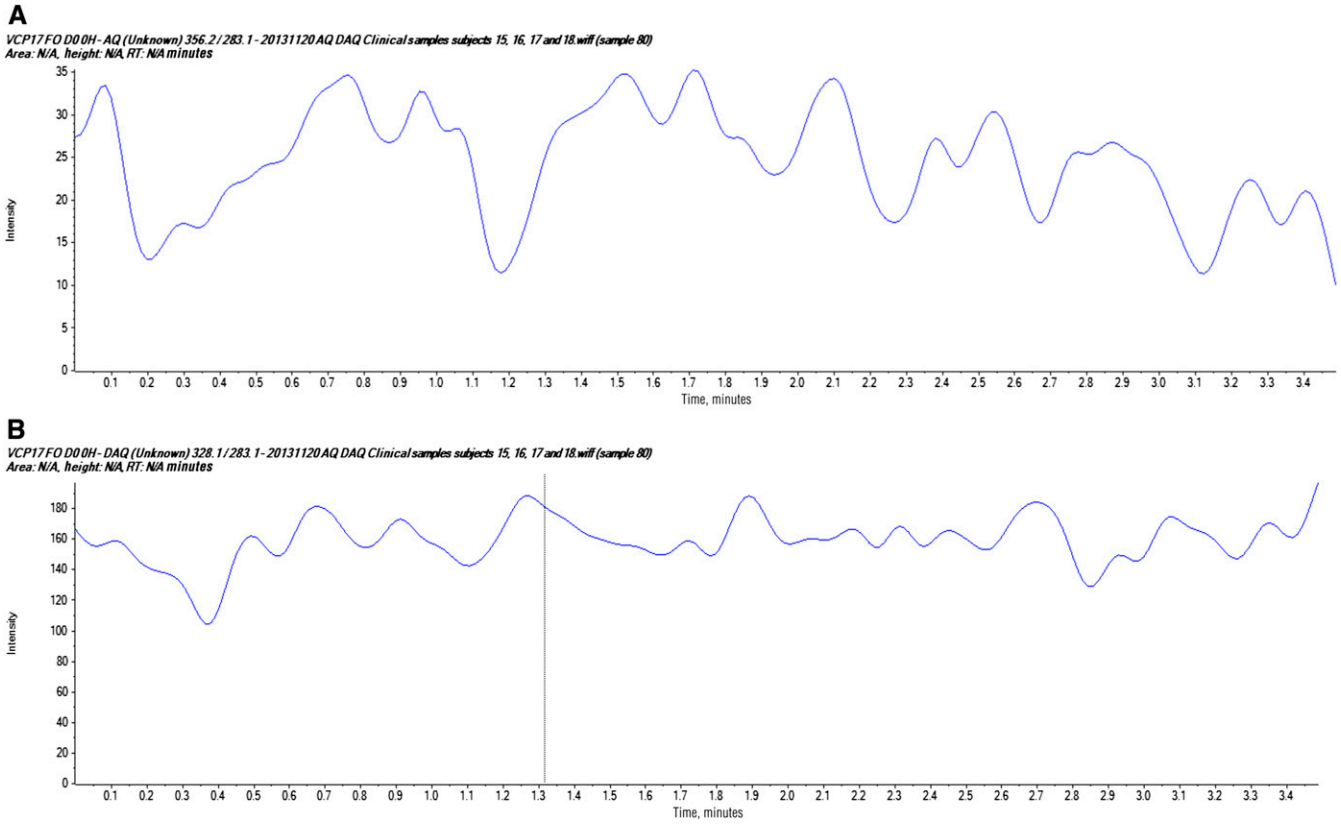
SUPPLEMENTAL FIGURE 2. Quality control (QC) low-range plasma sample (AQ 2.34 ng/mL/DAQ 6.00 ng/mL). (**A**) QC low AQ. (**B**) QC low DAQ. AQ = amodiaquine; DAQ = desethylamodiaquine.



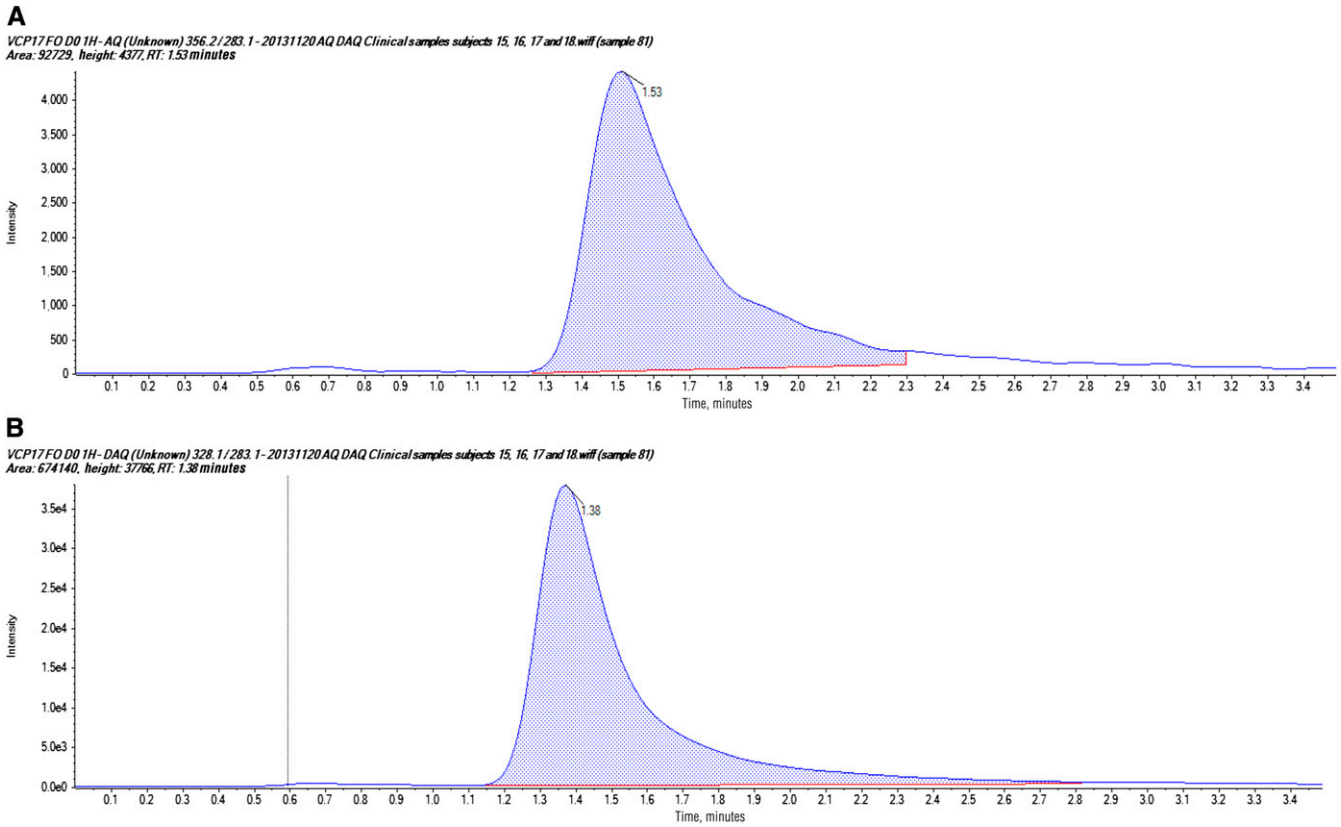
SUPPLEMENTAL FIGURE 3. Low calibration standard (AQ 0.391 ng/mL/DAQ 3.91 ng/mL). (**A**) AQ. (**B**) DAQ. AQ = amodiaquine; DAQ = desethylamodiaquine.



SUPPLEMENTAL FIGURE 4. High calibration standard (AQ 100 ng/mL/DAQ 2,000 ng/mL). (**A**) AQ. (**B**) DAQ. AQ = amodiaquine; DAQ = desethylamodiaquine.



SUPPLEMENTAL FIGURE 5. Subject VCP17FO plasma sample pre-dose. (**A**) AQ. (**B**) DAQ. AQ = amodiaquine; DAQ = desethylamodiaquine.



SUPPLEMENTAL FIGURE 6. Subject VCP17FO plasma sample 1 hour post-dose. (**A**) AQ (23.31 ng/mL). (**B**) DAQ (186.57 ng/mL). AQ = amodiaquine; DAQ = desethylamodiaquine.

Method validation. *Linearity and reproducibility.*

- Amodiaquine and DAQ inter-day accuracy and precision.

Calibration standards of plasma AQ/DAQ concentrations from 0.391/3.91 to 100/2,000 ng/mL were analyzed over the period of the study analysis (Supplemental Table 1). Good linearity was obtained over this range, with good precision and accuracy.

- For AQ, the LLOQ using 0.05 mL of plasma was 0.391 ng/mL with a CV of 3.46% and an inaccuracy of –0.62%. The quadratic regression equation $y = ax^2 + bx + c$, where x is the amount of drug and y is the ratio of AQ or DAQ area to internal standard area, was used to determine the concentrations of unknowns and QC samples. A typical regression equation of a calibration curve for AQ was $y = 2.3011e^{-4}x^2 + 0.1297x + 0.00454$, where $r^2 = 0.9981$.
- For DAQ, the LLOQ using 0.05 mL of plasma was 3.91 ng/mL with a CV of 1.20% and an inaccuracy of 1.55%. A typical regression equation of a calibration curve was $y = 3.4211e^{-6}x^2 + 0.1526x + 6.7194$, where $r^2 = 0.9991$.

SUPPLEMENTAL TABLE 1
Inter-day accuracy and precision of AQ/DAQ assay

	Mean (ng/mL)	SD (ng/mL)	CV (%)	*n*	Accuracy (%)
AQ nominal concentration (ng/mL)					
0.391	0.39	0.01	3.46	7	99.38
0.781	0.77	0.02	3.04	10	98.85
1.56	1.57	0.08	5.27	10	100.38
3.13	3.11	0.14	4.55	10	99.42
6.25	6.23	0.35	5.56	10	99.60
12.5	12.56	0.81	6.42	10	96.86
25.0	25.08	1.23	4.91	10	100.33
50.0	50.06	2.71	5.41	10	100.11
100	99.64	0.77	0.78	10	99.64
DAQ nominal concentration (ng/mL)					
3.91	3.97	0.05	1.20	10	101.55
7.81	7.39	0.22	2.94	9	94.64
15.6	15.11	0.54	3.58	10	96.87
31.3	31.19	1.21	3.88	10	99.66
62.5	62.53	2.38	3.80	10	100.05
125	129.24	2.99	2.32	10	103.40
250	252.87	12.90	5.10	10	101.15
500	506.71	25.27	4.99	10	101.34
1,000	980.58	67.78	6.91	10	98.06
2,000	1,993.44	23.37	1.17	10	99.67

AQ = amodiaquine; CV = coefficient of variation; DAQ = desethylamodiaquine.

- Amodiaquine and DAQ intraday accuracy and precision

Calibration standards of plasma AQ and DAQ were analyzed in quintuplicate on a single day (see Supplemental Table 2). Good linearity was obtained over this range, with good precision and accuracy.

SUPPLEMENTAL TABLE 2
Intraday accuracy and precision of DA/DAQ assay

	Mean (ng/mL)	SD (ng/mL)	CV (%)	*n*	Accuracy (%)
AQ nominal concentration (ng/mL)					
0.391	0.40	0.01	3.28	5	101.79
0.781	0.78	0.06	7.90	5	99.87
1.56	1.45	0.04	2.74	5	92.82
3.13	3.02	0.13	4.29	5	96.36
6.25	6.36	0.18	2.79	5	101.82
12.5	12.95	0.56	4.29	5	103.62
25.0	25.79	0.59	2.28	5	103.16
50.0	51.14	1.59	3.11	5	102.28
100	97.96	1.03	1.06	5	97.86
DAQ nominal concentration (ng/mL)					
3.91	3.86	0.10	2.60	5	98.67
7.81	7.74	0.12	1.61	5	99.08
15.6	15.49	0.61	3.94	5	99.27
31.3	31.15	0.84	2.70	5	99.52
62.5	61.66	1.81	2.94	5	98.66
125	126.57	2.10	1.66	5	101.26
250	252.61	9.47	3.75	5	101.04
500	499.39	24.55	4.92	5	99.88
1,000	1,011.95	27.43	2.71	5	101.20
2,000	1,985.18	47.33	2.38	5	99.26

AQ = amodiaquine; CV = coefficient of variation; DAQ = desethylamodiaquine.

Quality control plasma samples.

- Accuracy and precision of QC samples

Results of accuracy and precision of QC samples (low–mid–high concentrations) assayed are shown for AQ and DAQ in Supplemental Table 3.

SUPPLEMENTAL TABLE 3
Accuracy and precision of AQ/DAQ QC samples

	Nominal (ng/mL)	Mean (ng/mL)	SD (ng/mL)	CV (%)	*n*	Accuracy (%)
AQ						
QC low	2.34	2.51	0.17	6.62	20	107.44
QC mid	37.50	39.11	1.40	3.59	21	104.29
QC high	75.00	75.22	2.22	2.95	21	100.30
DAQ						
QC low	6	6.40	0.26	4.14	21	106.65
QC mid	750	781.90	26.89	3.44	21	104.25
QC high	1,500	1,513.24	31.81	2.10	21	100.88

AQ = amodiaquine; CV = coefficient of variation; DAQ = desethylamodiaquine; QC = quality control.

Uncertainty of measurement of plasma AQ and DAQ. Uncertainty of measurement estimate for Plasma AQ is at the following concentrations:

- 2.34 ng/mL: 8.74% (2.14–2.55 ng/mL)
- 37.5 mL: 7.23% (34.79–44.73 ng/mL)
- 75 g/mL: 6.06% (70.45–79.55 ng/mL)

Uncertainty of measurement estimate for Plasma DAQ is at the following concentrations:

- 6 ng/mL: 8.24% (5.5–6.5 ng/mL)
- 750 ng/mL: 7.03% (697–803 ng/mL)
- 1,500 ng/mL: 6.32% (1,405–1,595 ng/mL)

SUPPLEMENTAL MATERIAL 3

LC/MS/MS ANALYSIS OF ARN

Standards.

- Artemisinin. Worldwide Antimalarial Resistance Network Reference compound.
- Internal standard, SIL DHA. Worldwide Antimalarial Resistance Network Reference compound.

Chromatographic conditions.

- Liquid chromatography column: Hypersil Gold C18 100 mm × 2.1 mm I.D. 5 µm, 25005-102130.
- Pre-column: Hypersil Gold C18 10 mm × 2.1 mm I.D. 3 µm, 25005-012101 (changed every 200 samples or earlier if needed).
- Mobile phase flow: 0.5 mL/minute.
- Run time: 3 minutes.
- Mobile phase A: ACN–ammonium acetate 10 mM pH 3.5 (50–50, v/v).
- Mobile phase B: Methanol–ACN (75–25, v/v).

Gradient:

Total time (minutes)	A (%)	B (%)
0	60	40
0.3	60	40
0.6	0	100
1.4	0	100
1.8	60	40
3	0	100

Mass spectrometer conditions:

Curtain gas	25
CAD gas	High
IS (V)	5,500
Temp (°C)	300
GS1	50
GS2	50
IHE	On

Mass spectrometry transitions:

Analyte	Q1 (Da)	Q3 (Da)	Time (ms)	DP (v)	EP (v)	CE (v)	CXP (v)
ARN	283.088	209.4	150	66	10	9	18
SIL DHA	307.0	168.0	150	36	10	23	12

ARN = artemisinin; DHA = dihydroartemisinin; SIL = stable isotope labeled.

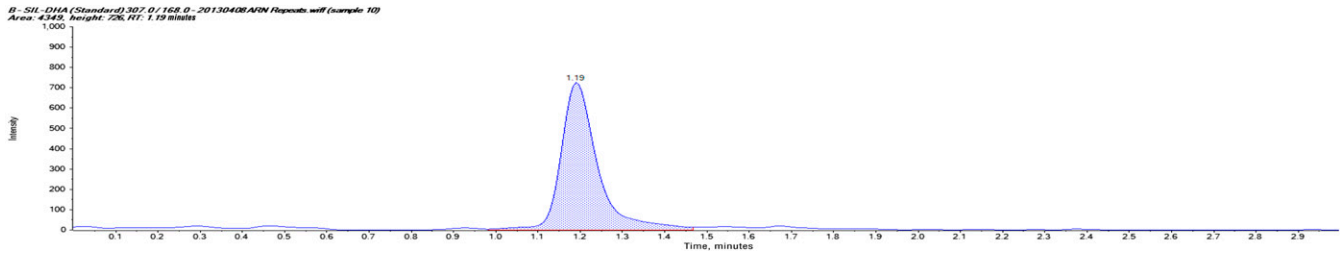
Subject plasma sample preparation.

- Calibrators and QCs were prepared by spiking ARN-free citrate phosphate dextrose anticoagulated human plasma (50 µL) with ACN containing internal standard (2 ng/mL SIL DHA in ACN; 200 µL) or blank ACN (200 µL) for the double blank. Calibrators and blanks were then treated as unknowns.
- Subject plasma samples were thawed in a room-temperature circulating water bath and were vortex mixed before analysis.
- Subject or spiked plasma (50 µL) was added to pre-labeled 1.5-mL polypropylene microfuge tubes. To these tubes was added internal standard solution (2 ng/mL SIL DHA in ACN; 200 µL), and the tubes were mixed by vortexing for 1 minute at maximum vortex setting.
- The tubes were then centrifuged (20,817 × *g*, 5 minutes at 4°C).
- The organic layer (100 µL) was subsequently transferred to a 96-well polypropylene microtitre plate containing 100 µL mobile phase A.
- Inject 10 µL onto the column.

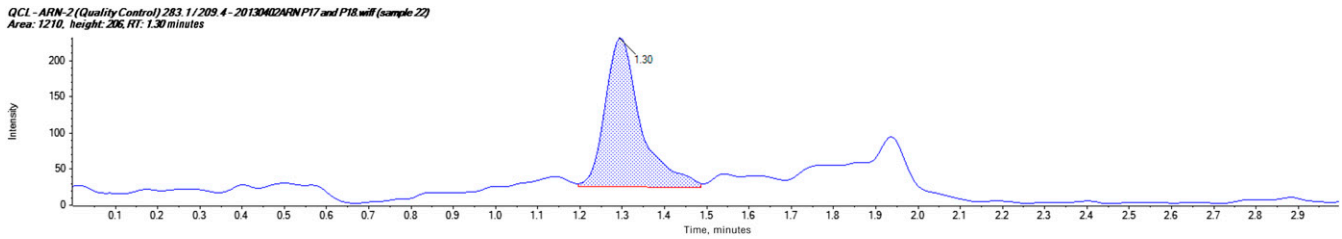
Calculation of results. Chromatograms were integrated and processed using Applied Biosystems software (Analyst V1.4.2). Concentrations of ARN in subject plasma samples and QCs were calculated from regression curves of peak area ratios (ARN:SIL DHA) versus analyte concentration using Multiquant 2.1 software. A weighting of ($1/x^2$) was used to generate a quadratic regression curve.

Testing of drug-free plasmas for ion suppression. Six drug-free plasma samples were tested for matrix effect (ion suppression) to identify a drug-free plasma that showed no identifiable effect on drug quantification. This was performed by infusing a low quantity of ARN (10 ng/mL) while injecting ACN extracts from the six drug-free plasmas. None of the plasmas showed ion suppression and one of these, 2HSB003/09, was used to prepare standards and QC samples.

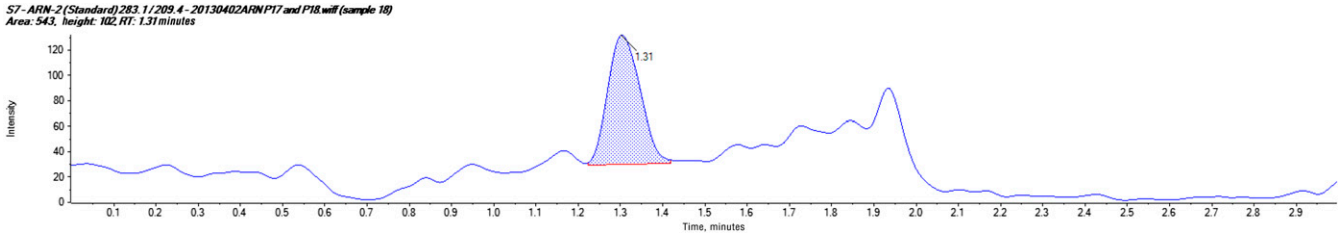
Chromatograms. Typical chromatograms of ARN and the internal standard SIL DHA in plasma are shown in Supplemental Figures 7–12. They are internal standard SIL DHA (Supplemental Figure 7); low-range QC plasma ARN (Supplemental Figure 8); a low calibration standard (Supplemental Figure 9); a high calibration standard (Supplemental Figure 10); subject plasma at time = 0 hour (Supplemental Figure 11); subject plasma at time = 1 hour (Supplemental Figure 12). The retention times for ARN and SIL DHA were approximately 1.30 and 1.19 minutes, respectively.



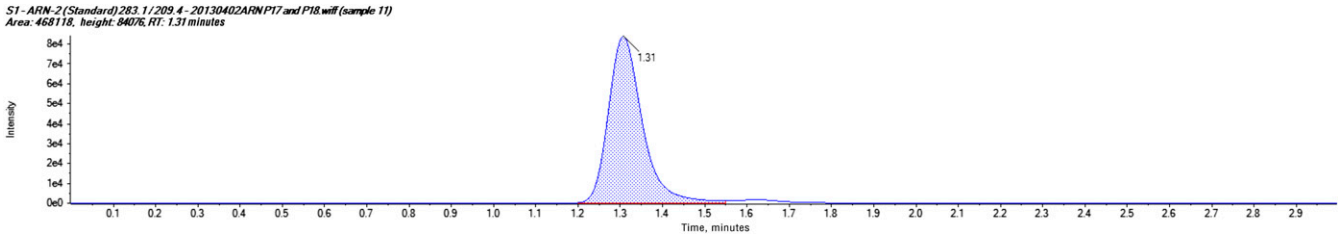
SUPPLEMENTAL FIGURE 7. Internal standard SIL DHA. DHA = dihydroartemisinin; SIL = stable isotope labeled.



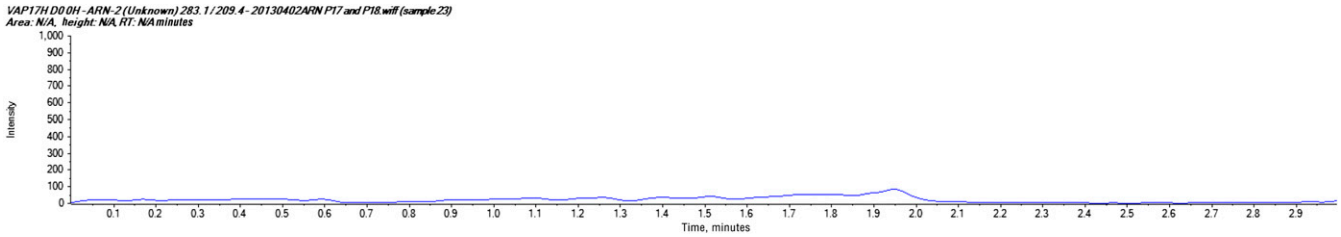
SUPPLEMENTAL FIGURE 8. Quality control (QC) low-range plasma sample QC (ARN 2 ng/mL). ARN = artemisinin.



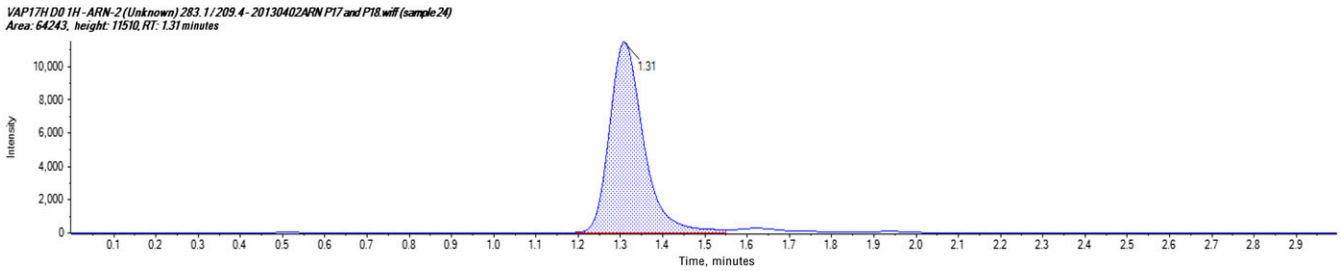
SUPPLEMENTAL FIGURE 9. Low calibration standard (ARN 1 ng/mL). ARN = artemisinin.



SUPPLEMENTAL FIGURE 10. High calibration standard (ARN 1,000 ng/mL). ARN = artemisinin.



SUPPLEMENTAL FIGURE 11. Subject VAP17H plasma at time = 0 hour.



SUPPLEMENTAL FIGURE 12. Subject VAP17H plasma at time = 1 hour (109.32 ng/mL).

Method validation. *Linearity and reproducibility.*

- **Artemisinin inter-day accuracy and precision**

Calibration standards of plasma ARN concentrations from 1 to 1,000 ng/mL analyzed over the period of the study analysis. Good linearity was obtained over this range, with good precision and accuracy.

SUPPLEMENTAL TABLE 4
Inter-day accuracy and precision of ARN assay

ARN nominal concentration (ng/mL)	Mean (ng/mL)	SD (ng/mL)	CV%	*n*	Accuracy (%)
1,000	1,050.61	85.80	8.17	21	105.06
400	339.95	19.20	5.65	11	84.99
100	99.71	4.73	4.75	11	99.71
40	41.86	3.00	7.17	10	104.65
10	10.08	0.56	5.52	11	100.79
2.5	2.48	0.14	5.73	11	99.38
1	1.04	0.13	12.26	18	104.44

ARN = artemisinin; CV = coefficient of variation.

For ARN, the LLOQ using 0.05 mL of plasma was 1 ng/mL with a CV of 12.26% and an inaccuracy of 4.44%. The quadratic regression equation $y = ax^2 + bx + c$, where x is the amount of drug and y is the ratio of ARN area to internal standard area, was used to determine the concentrations of unknowns and QC samples. A typical regression equation of a calibration curve was $y = -5.071x^2 + 0.254x + 0.0208$, where $r^2 = 0.9995$.

- **Artemisinin intraday accuracy and precision**

Calibration standards of plasma ARN were analyzed in quintuplicate on a single day (Supplemental Table 5). Good linearity was obtained over this range, with good precision and accuracy.

SUPPLEMENTAL TABLE 5
Intraday accuracy and precision of ARN assay

ARN nominal concentration (ng/mL)	Mean (ng/mL)	SD (ng/mL)	CV (%)	*n*	Accuracy (%)
1,000	913.37	34.65	3.79	5	91.34
400	349.81	24.72	7.07	5	87.45
100	105.37	5.30	5.03	5	105.37
40	41.92	1.60	3.81	5	104.81
10	9.82	0.50	5.09	5	98.24
2.5	2.46	0.16	6.56	5	98.48
1	1.06	0.09	8.11	5	106.20

ARN = artemisinin; CV = coefficient of variation.

Accuracy and precision of QC samples.

- Results of accuracy and precision of QC samples (low–mid–high concentrations) assayed are shown for ARN in Supplemental Table 6.

SUPPLEMENTAL TABLE 6
Accuracy and precision of ARN QC samples

ARN	Nominal (ng/mL)	Mean (ng/mL)	SD (ng/mL)	CV (%)	*n*	Accuracy (%)
QC High	500	462.59	37.39	8.08	22	92.52
QC Mid	20	18.22	0.75	4.09	22	91.11
QC Low	2	1.95	0.14	7.12	22	97.43

ARN = artemisinin; CV = coefficient of variation; QC = quality control.

SUPPLEMENTAL MATERIAL 4

LC/MS/MS ANALYSIS OF ARS AND DHA

Standards.

- Artesunate, Lot no. MB17174, Manufacture Code 256283AD.
- Dihydroartemisinin, Lot no. DHN060306 (Supplied by DK Pharma, Hanoi, Vietnam).
- Internal standards, SIL ARS and SIL DHA. Worldwide Antimalarial Resistance Network Reference compound.

Chromatographic conditions.

- Column: Thermo Fisher Hypersil Gold (100 mm × 2.1 mm; 5 µm).
- Run time: 5.5 minutes.
- Mobile phase A: 5 mM NH_4 acetate, 0.5% acetic acid, and 50% ACN.
- Mobile phase B: ACN.

Gradient:

Time (minutes)	Module	Events	Parameter
0.30	Pumps	Pump B concentration	0.0
0.80	Pumps	Pump B concentration	100
2.20	Pumps	Pump B concentration	100
2.40	Pumps	Total flow	1
3.30	Pumps	Total flow	1
3.50	Pumps	Pump B concentration	0
3.50	Pumps	Total flow	0.75
5.20	Pumps	Pump B concentration	0.0
5.40	Pumps	Total flow	0.5
5.50	System controller	Stop	–

Mass spectrometer conditions:

Curtain gas	25
CAD gas	High
IS (V)	5,500
Temp (°C)	300
GS1	50
GS2	50
IHE	On

Mass spectrometry transitions:

Analyte	Q1 (Da)	Q3 (Da)	Time (ms)	DP (v)	EP (v)	CE (v)	CXP (v)
ARS	402.1	267.1	100	46	10	15	22
DHA	302.2	163.2	100	41	10	23	14
SIL ARS	406.0	163.0	100	40	10	16	12
SIL DHA	307.0	168.0	00	36	10	23	12

ARS = artesunate; DHA = dihydroartemisinin; SIL = stable isotope labeled.

Subject plasma sample preparation.

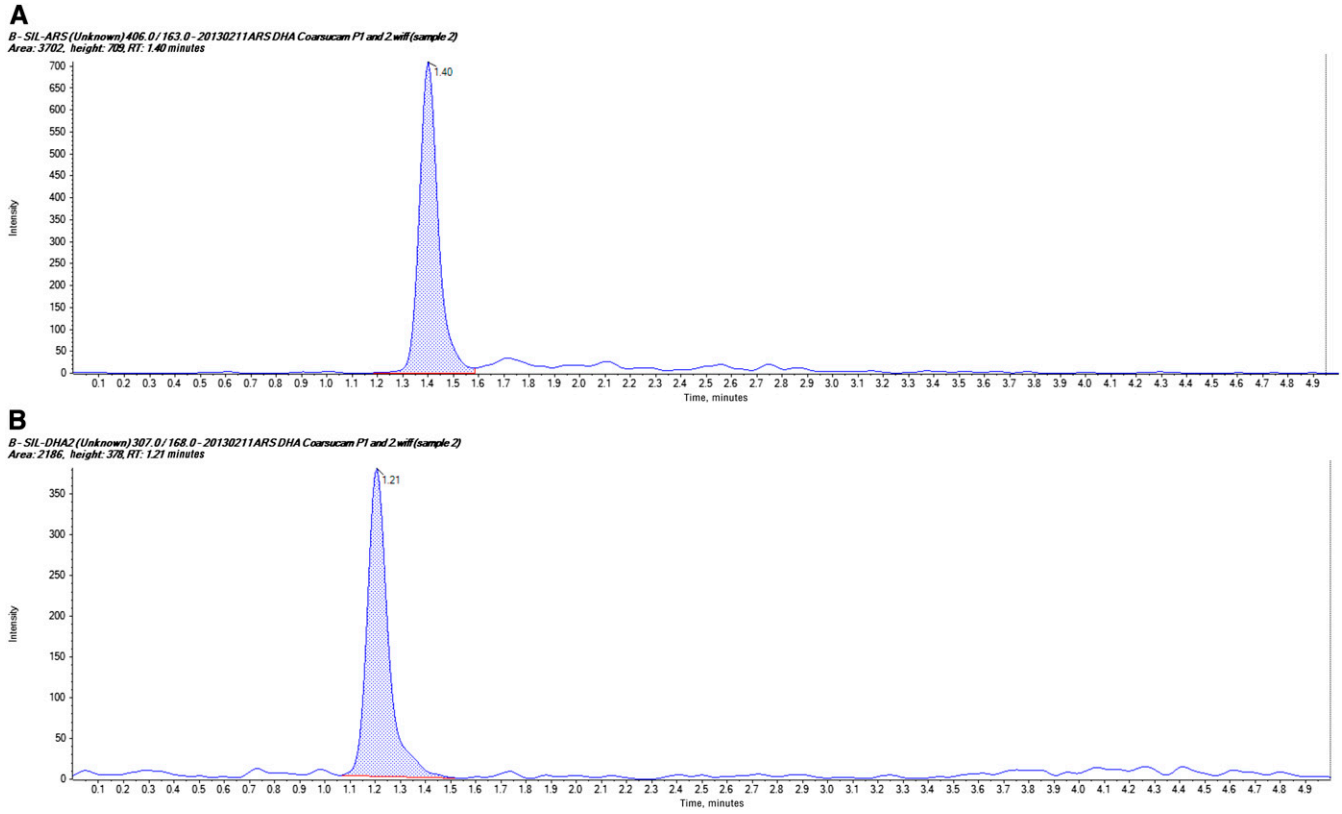
- Calibrators and QCs were prepared by spiking ARS- and DHA-free lithium heparin anticoagulated human plasma (50 µL) with ACN containing internal standards (2 ng/mL SIL ARS and 10 ng/mL SIL DHA in ACN; 200 µL) or blank ACN (200 µL) for the double blank. Calibrators and blanks were then treated as unknowns.
- Subject plasma samples were thawed in a room-temperature circulating water bath and were vortex mixed before analysis.
- Subject or spiked plasma (50 µL) was added to pre-labeled 1.5-mL polypropylene microfuge tubes. To these tubes was added an internal standard solution (2 ng/mL SIL ARS and 10 ng/mL SIL DHA in ACN; 200 µL), and the tubes were mixed by vortexing for 1 minute at maximum vortex setting.
- The tubes were then centrifuged (20,817 × *g*, 5 minutes at 4°C).
- The organic layer (100 µL) was subsequently transferred to a 96-well polypropylene microtitre plate containing 100 µL of 0.1% formic acid and the assay plate was sealed with a silicone mat.
- Inject 10 µL onto the column.

Calculation of results. Chromatograms were integrated and processed using Applied Biosystems software (Analyst V1.4.2). Concentrations of DHA and ARS in subject plasma samples and QCs were calculated from regression curves of peak area ratios (DHA:SIL DHA and ARS:SIL ARS) versus analyte concentration using Multiquant 2.1 software. A weighting of ($1/x^2$) was used to generate a quadratic regression curve.

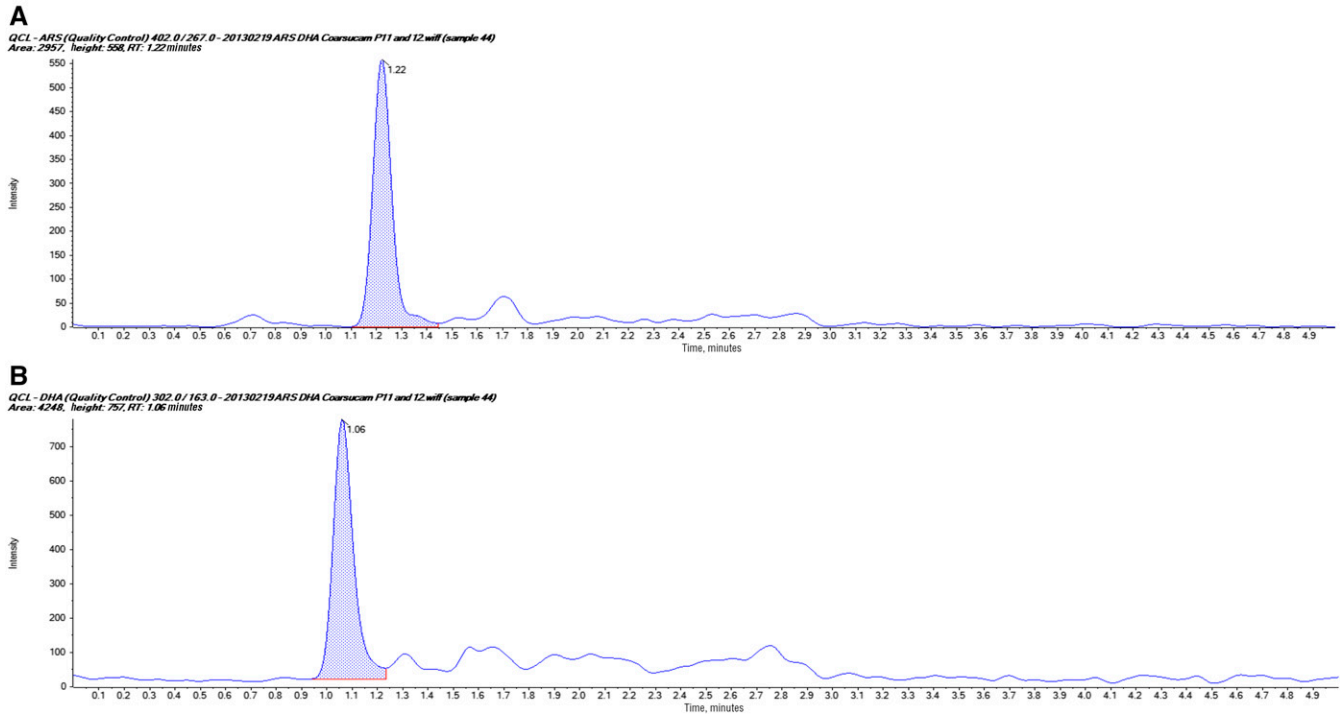
Testing of drug-free plasmas for ion suppression. Six drug-free plasma samples were tested for matrix effect (ion suppression) to identify a drug-free plasma that showed no identifiable effect on drug quantification. This was performed by infusing a low quantity of AS (10 ng/mL) and DHA (10 ng/mL) while injecting mobile phase extracts from the six drug-free plasmas. None of the plasmas showed ion suppression and one of these, 2HSB003/09, was used to prepare standards and QC samples.

Chromatograms.

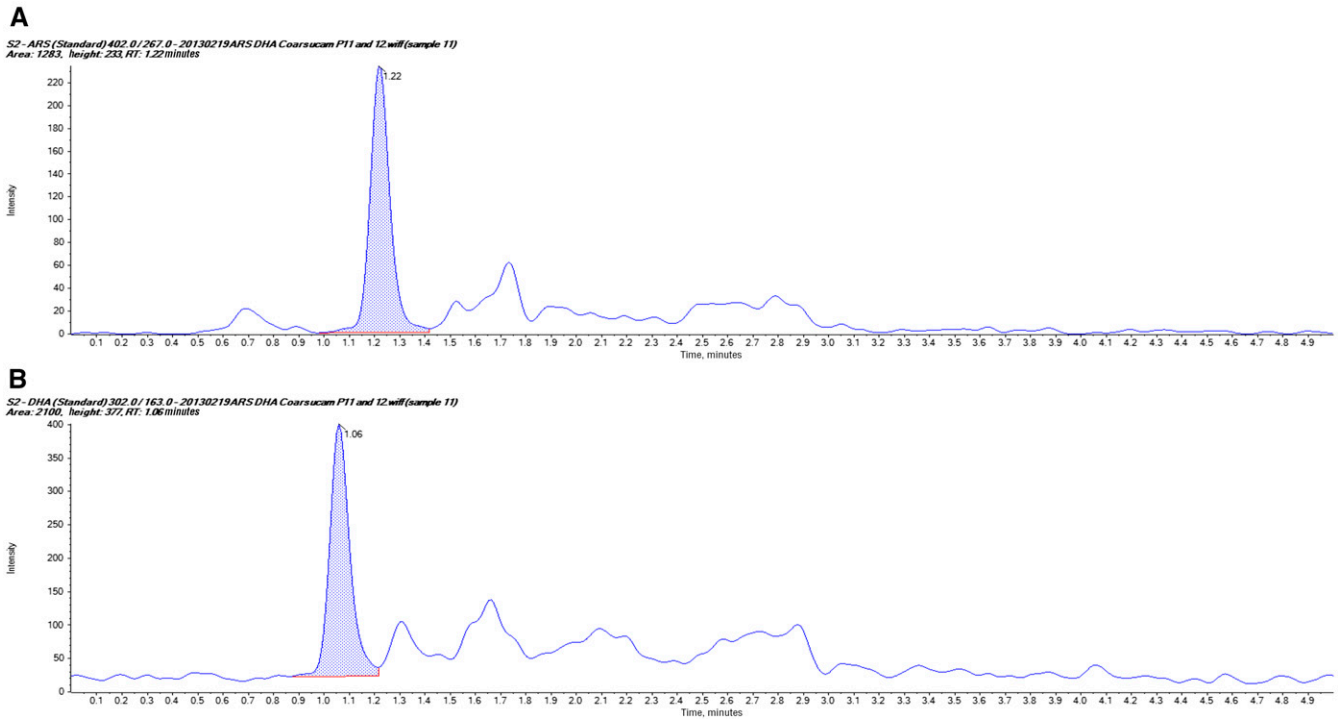
- Typical chromatograms of ARS, DHA, and the internal standards SIL ARS and SIL DHA in plasma are shown in Supplemental Figures 13–18. They are internal standards SIL ARS and SIL DHA (Supplemental Figure 13); low-range QC plasma ARS and DHA (Supplemental Figure 14); a low calibration standard (Supplemental Figure 15); a high calibration standard (Supplemental Figure 16); subject plasma sample pre-dose (Supplemental Figure 17); subject plasma sample at 1 hour post-dose (Supplemental Figure 18).
- The retention times for ARS, DHA, SIL ARS, and SIL DHA were approximately 1.22, 1.06, 1.40, and 1.21 minutes, respectively.



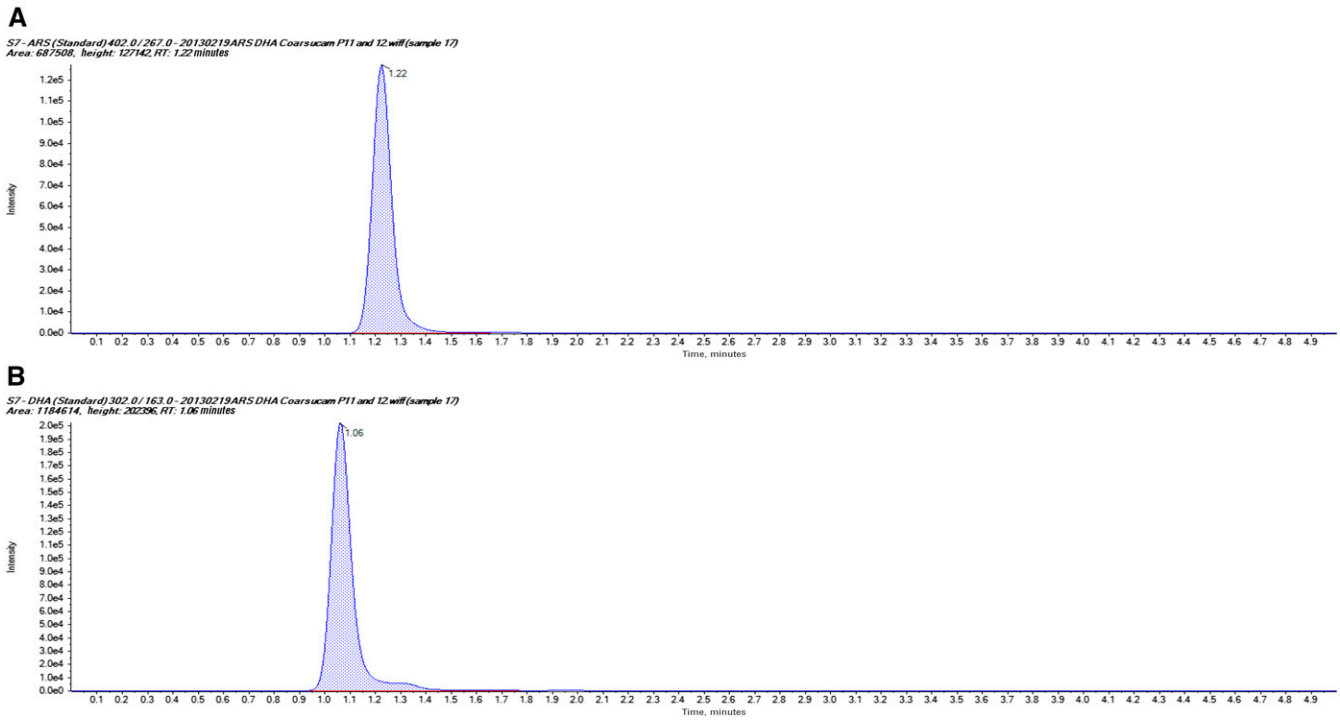
SUPPLEMENTAL FIGURE 13. Internal standards of SIL ARS and SIL dihydroartemisinin (DHA). (**A**) SIL ARS (2 ng/mL). (**B**) SIL DHA (10 ng/mL). ARS = artesunate; SIL = stable isotope labeled.



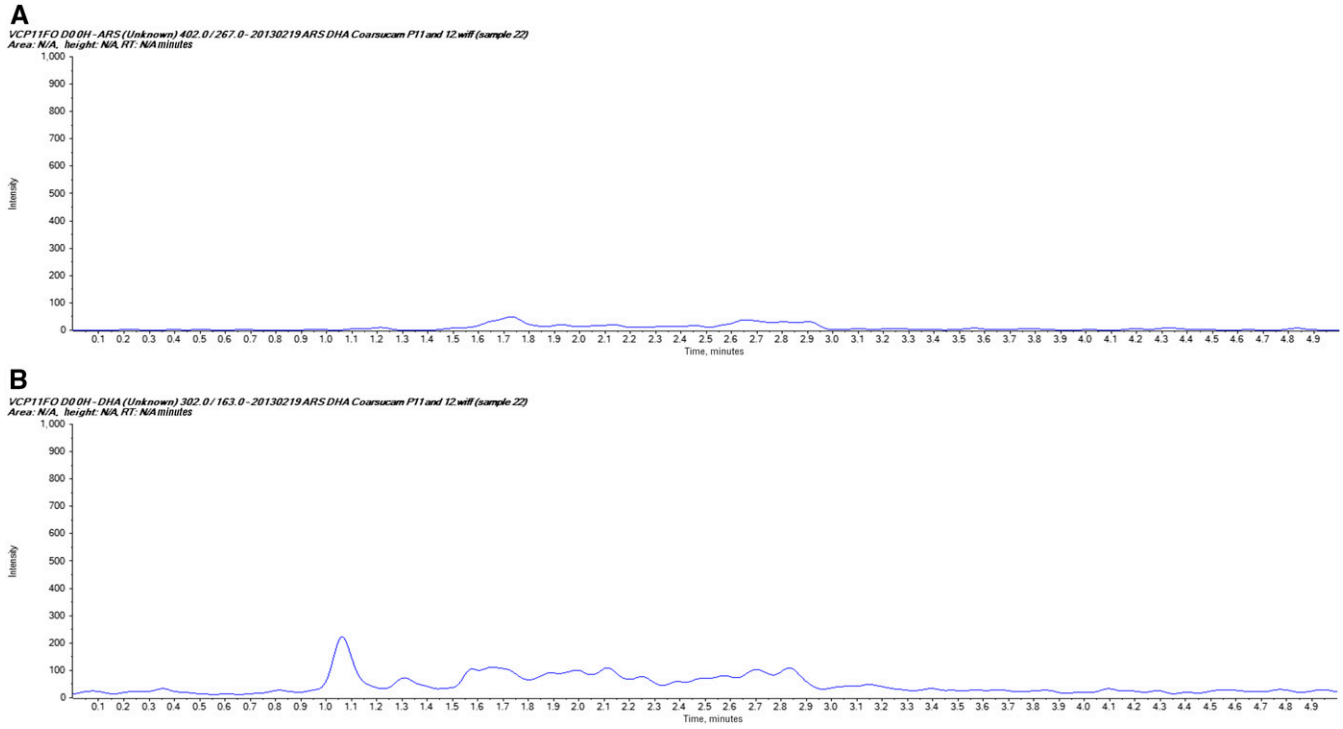
SUPPLEMENTAL FIGURE 14. Quality control (QC) low-range plasma sample (ARS 2.9 ng/mL; dihydroartemisinin (DHA) 5.87 ng/mL). (**A**) QC low ARS. (**B**) QC low DHA. ARS = artesunate.



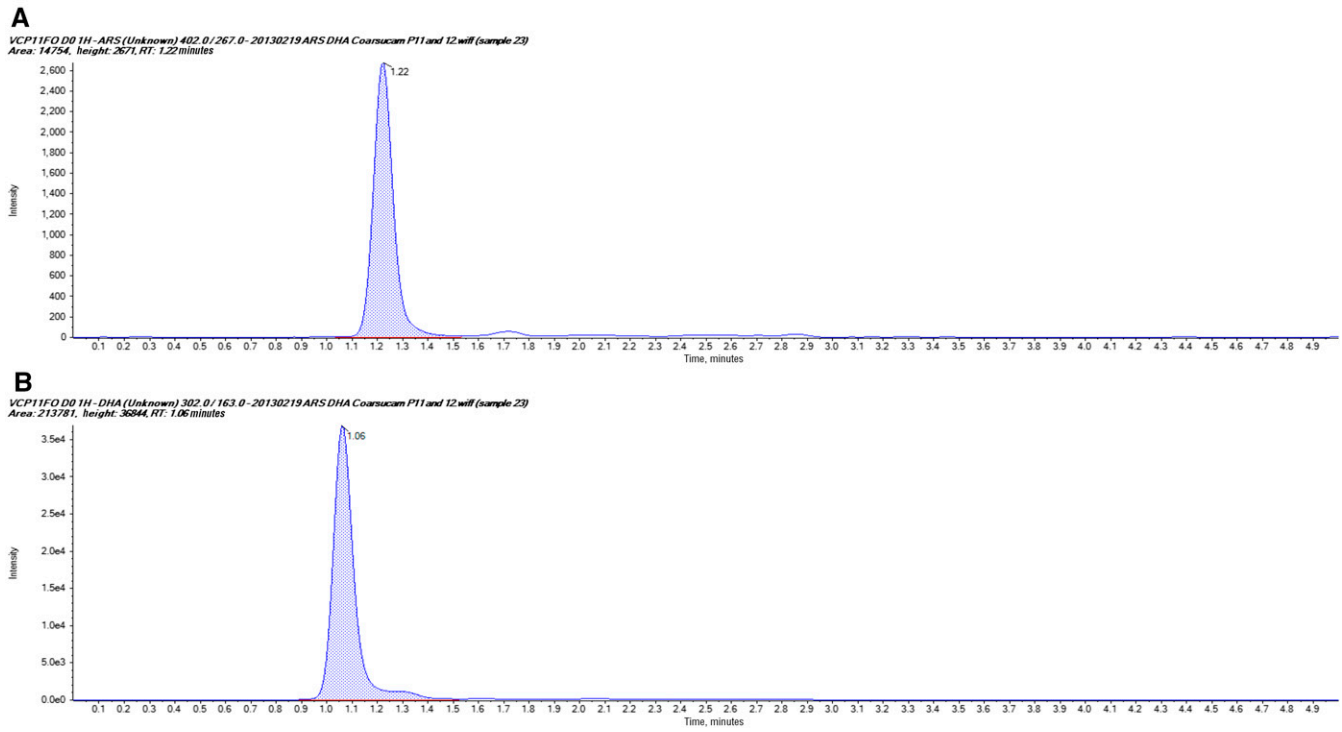
SUPPLEMENTAL FIGURE 15. Low calibration standard (ARS 1.19 ng/mL; dihydroartemisinin (DHA) 1.96 ng/mL). (**A**) ARS. (**B**) DHA. ARS = artesunate.



SUPPLEMENTAL FIGURE 16. High calibration standard (ARS 728 ng/mL; dihydroartemisinin (DHA) 2,500 ng/mL). (**A**) ARS. (**B**) DHA. ARS = artesunate.



SUPPLEMENTAL FIGURE 17. Subject VCP11FO plasma sample pre-dose. (**A**) ARS. (**B**) dihydroartemisinin (DHA). ARS = artesunate.



SUPPLEMENTAL FIGURE 18. Subject VCP11FO plasma sample 1 hour post-dose. (**A**) ARS. (**B**) dihydroartemisinin (DHA). ARS = artesunate.

Method validation. *Linearity and reproducibility.*

- **Artesunate and DHA inter-day accuracy and precision**

Calibration standards of plasma ARS and DHA concentrations from 1.19/1.96 to 728/2,500 ng/mL were analyzed over the period of the study (Supplemental Table 7). Good linearity was obtained over this range, with good precision and accuracy.

SUPPLEMENTAL TABLE 7

Inter-day accuracy and precision of ARS/DHA assay

	Mean (ng/mL)	SD (ng/mL)	CV (%)	*n*	Accuracy (%)
ARS nominal concentration (ng/mL)					
1.19	1.19	0.10	8.65	23	99.89
3.86	4.00	0.20	4.99	17	103.66
21.2	20.81	1.36	6.52	17	98.18
69	67.67	3.96	5.86	17	98.07
224	223.57	11.01	4.92	17	99.81
728	729.92	23.79	3.26	28	100.26
DHA nominal concentration (ng/mL)					
1.96	1.90	0.28	14.48	21	97.06
7.82	7.97	0.87	10.89	16	101.94
39.1	39.30	2.49	6.34	17	100.51
156	156.29	10.28	6.58	16	100.19
625	632.62	62.46	9.87	17	101.22
2,500	2,499.08	93.88	3.76	27	99.96

ARS = artesunate; CV = coefficient of variation; DHA = dihydroartemisinin.

1. For ARS, the LLOQ using 0.05 mL of plasma was 1.19 ng/mL with a CV of 8.65% and an inaccuracy of –0.11%. The quadratic regression equation $y = ax^2 + bx + c$, where x is the amount of drug and y is the ratio of ARS or DHA area to internal standard area, was used to determine the concentrations of unknowns and QC samples. A typical regression equation of a calibration curve was $y = -5.071\ x^2 + 0.254\ x + 0.0208$, where $r^2 = 0.9995$.
2. For DHA, the LLOQ using 0.05 mL of plasma was 1.96 ng/mL with a CV of 14.48% and an inaccuracy of –2.94%. A typical regression equation of a calibration curve was $y = -1.427e^{-5}x^2 + 0.3065x + 0.5201$, where $r^2 = 0.9978$.

- **Artesunate and DHA intraday accuracy and precision**

Calibration standards of plasma ARS and DHA were analyzed in quintuplicate on a single day (Supplemental Table 8). Good linearity was obtained over this range, with good precision and accuracy.

SUPPLEMENTAL TABLE 8

Intraday accuracy and precision of ARS/DHA assay

	Mean (ng/mL)	SD (ng/mL)	CV (%)	*n*	Accuracy (%)
ARS nominal concentration (ng/mL)					
1.19	1.22	0.03	2.55	5	102.69
3.86	3.91	0.23	6.00	5	101.30
21.2	20.92	1.44	6.88	5	98.68
69	66.07	2.37	3.59	5	95.75
224	228.46	10.90	4.77	5	101.99
728	726.30	11.09	1.53	5	99.77
DHA nominal concentration (ng/mL)					
1.96	1.97	0.15	7.54	5	100.31
7.82	8.33	0.40	4.83	5	106.55
39.1	38.52	2.77	7.20	5	98.51
156	153.34	4.99	3.25	5	98.30
625	591.28	38.93	6.58	5	94.61
2,500	2,545.61	45.38	1.78	5	101.82

ARS = artesunate; CV = coefficient of variation; DHA = dihydroartemisinin.

Quality control plasma samples.

- **Accuracy and precision of QC samples**

Results of accuracy and precision of QC samples (low–mid–high concentrations) assayed are shown for DHA and ARS in Supplemental Table 9.

SUPPLEMENTAL TABLE 9

Accuracy and precision of ARS/DHA QC samples

	Nominal (ng/mL)	Mean (ng/mL)	SD (ng/mL)	CV (%)	*n*	Accuracy (%)
ARS						
QC low	2.9	3.03	0.21	6.85	29	104.38
QC mid	51.7	51.28	3.25	6.35	29	99.18
QC high	546	552.43	30.02	5.43	29	101.18
DHA						
QC low	5.87	5.77	0.61	10.53	29	98.30
QC mid	117	120.69	11.69	9.68	29	103.15
QC high	1,880	1,871.77	134.88	7.21	29	99.56

ARS = artesunate; CV = coefficient of variation; DHA = dihydroartemisinin; QC = quality control.

SUPPLEMENTAL MATERIAL 5

LC/MS/MS ANALYSIS OF PPQ

Standards.

- Piperaquine as tetraphosphate salt. Worldwide Antimalarial Resistance Network Reference compound.
- Internal standard: [$^2H_6$]—PPQ (SIL PPQ). Worldwide Antimalarial Resistance Network Reference compound.

Chromatographic conditions.

- Column: Gemini C-18 NX 5µ column, 50 mm × 4.6 mm. (Phenomenex) with a Gemini C-18 NX 4 mm × 2 mm I.D. guard column (Phenomenex) maintained at 30°C in a column oven.
- The mobile phase consisted of an isocratic mix in the ratio 15:85 of pump A 2.5 M ammonium bicarbonate pH 10 and pump B ACN pumped at a combined flow rate of 0.5 mL/minute.
- Samples were maintained at 4°C in a cooled autosampler before injection.
- Needle rinse was 30% ACN with 1% formic acid.

Mass spectrometer conditions.

- Mass transitions were monitored using MRM in electrospray ionisation (ESI) positive mode at 535→288 and 535→260 for PPQ and 541→294 for SIL PPQ.
- Source conditions were DP 121v, EP10v, CE47v, CXP 18v, curtain gas 35, collision gas medium, ion source gas1 50, ion source gas2 45, ion spray voltage 5500, interface heater on, and temperature 600.
- Data were acquired using ABSciex Analyst® software v1.51.

Subject plasma sample preparation.

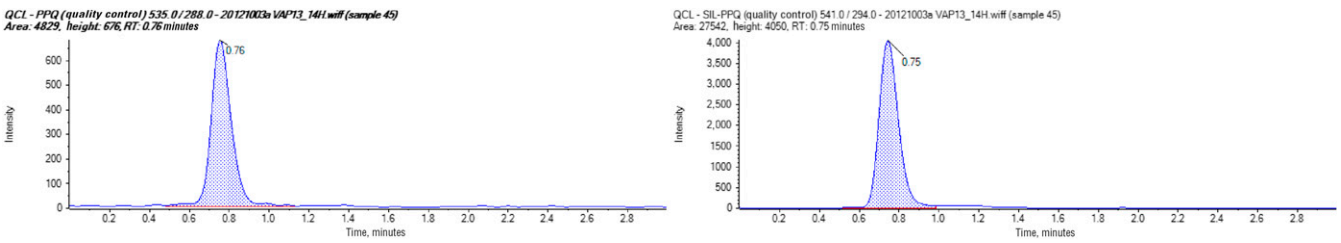
- In a 1.8-mL polypropylene microcentrifuge tube containing 50 µL of sample, spiked calibrator or QC plasma was added to 100 µL of 0.05 M carbonate buffer pH 10, and then 20 µL of internal standard (SIL PPQ IS2) was added and given a short vortex mix.
- Six hundred microliters of methyl-*tert*-butyl ether was added to each tube and vortex mixed for 1 minute. The tubes were centrifuged at 14,000 × *g* for 5 minutes in a refrigerated microcentrifuge set at 4°C.
- Four hundred and fifty microliters of supernatant was transferred to a clean 1.8-mL microcentrifuge tube and evaporated to dryness at 50°C under a stream of instrument-grade air.
- The residue was reconstituted with 100 µL of mobile phase and vortex mixed for 1 minute. The contents were transferred to a 96-well polypropylene analysis plate sealed with a silicone sealing mat and placed in the autosampler, 5 µL was injected on the column.

Calculation of results.

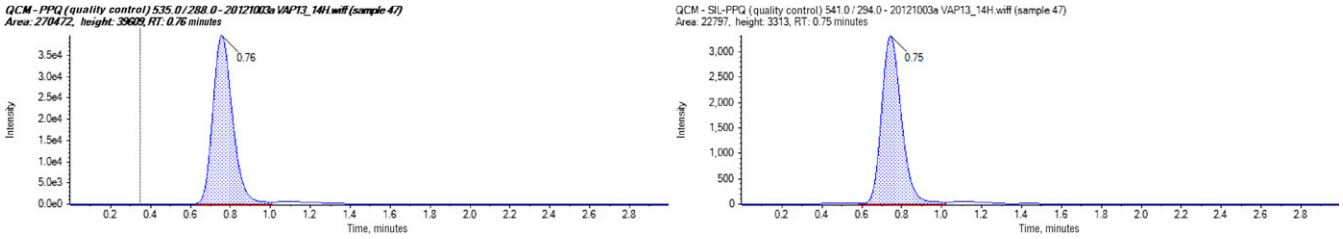
- Chromatograms were integrated and processed using the ABSciex Multiquant® software version 2.1.
- Concentrations of PPQ in plasma were calculated from quadratic regression curves of peak area ratios (PPQ:SIL PPQ) versus analyte concentration. A weighting of ($1/x^2$) was used.

Testing of drug-free plasmas for ion suppression. Six drug-free plasma samples were tested for matrix effect (ion suppression) to identify a drug-free plasma that showed no identifiable effect on drug quantitation. This was performed by infusing a low quantity of PPQ (10 ng/mL) while injecting mobile phase extracts from the six drug-free plasmas. None of the plasmas showed ion suppression and one of these, 2HSB003/09, was used to prepare standards and QC samples.

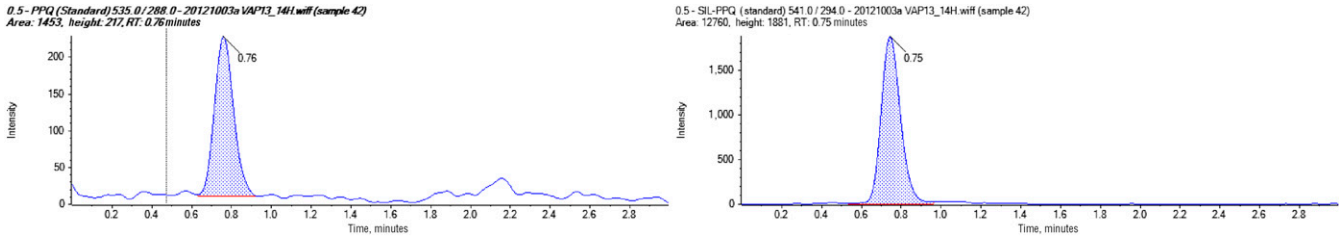
Chromatograms. Typical chromatograms of PPQ and the internal standard SIL PPQ in plasma are shown in Supplemental Figures 19–25. They are a low-range QC plasma nominal concentration PPQ 1 ng/mL (Supplemental Figure 19); a mid-range QC plasma nominal concentration PPQ 50 ng/mL (Supplemental Figure 20); a spiked DFP PPQ concentration 0.5 ng/mL (Supplemental Figure 21); a spiked DFP PPQ concentration 1,000 ng/mL (Supplemental Figure 22); plasma samples collected from a volunteer (VNAP02) on day 0 before dosing (Supplemental Figure 23); and VNAP02 plasma samples collected at 3 hours (Supplemental Figure 24) and 12 hours (Supplemental Figure 25) after the administration of Artequick (two tablets; 62.5–375 mg PPQ per tablets, daily for 3 days). The retention times for PPQ and SIL PPQ were approximately at 0.7 and 0.75 minutes, respectively.



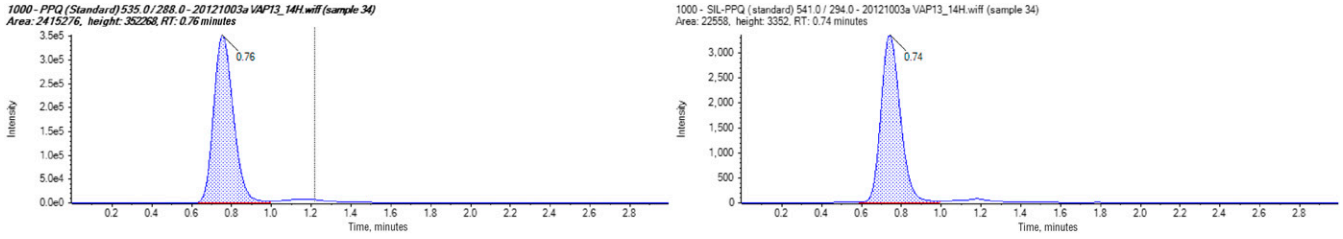
SUPPLEMENTAL FIGURE 19. Quality control (QC) low-range plasma sample (PPQ 1 ng/mL). PPQ = piperaquine.



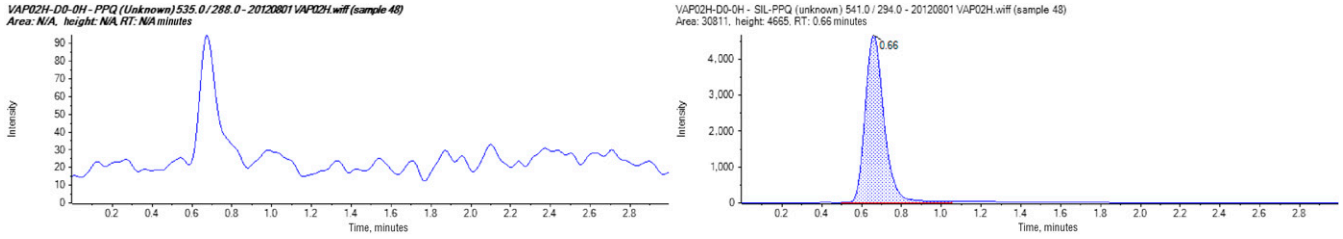
SUPPLEMENTAL FIGURE 20. Quality control (QC) mid-range plasma sample (PPQ 100 ng/mL). PPQ = piperaquine.



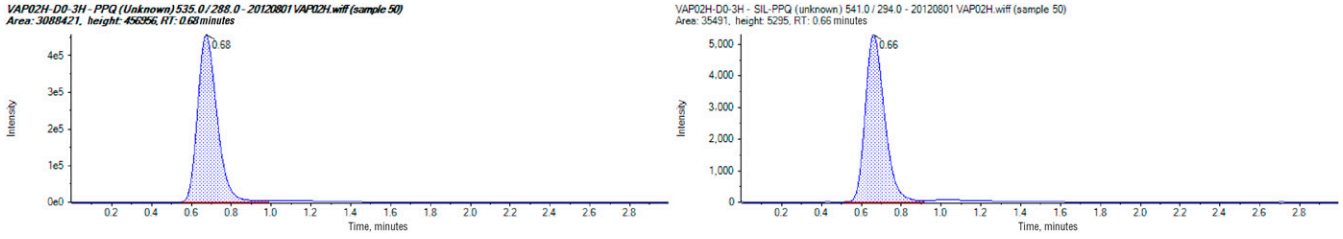
SUPPLEMENTAL FIGURE 21. Low calibration standard (PPQ 0.5 ng/mL). PPQ = piperaquine.



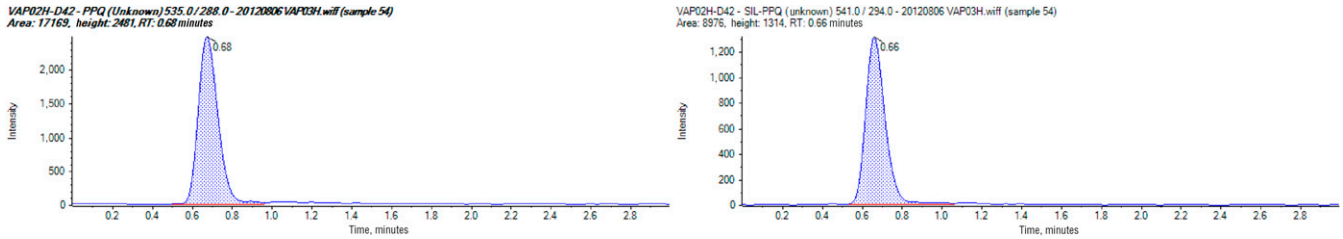
SUPPLEMENTAL FIGURE 22. High calibration standard (PPQ 1,000 ng/mL). PPQ = piperaquine.



SUPPLEMENTAL FIGURE 23. VAP02 plasma sample pre-dose.



SUPPLEMENTAL FIGURE 24. VAP02 plasma sample 3 hours post-dose (PPQ 882.5 ng/mL). PPQ = piperaquine.



SUPPLEMENTAL FIGURE 25. VAP02 plasma sample 12 hours post-dose (PPQ 841.0 ng/mL). PPQ = piperaquine.

Method validation.

- **Linearity and reproducibility**

Piperaquine inter-day precision of the assay calibration standards of plasma PPQ concentrations between 0.25 and 1,000 ng/mL were obtained over at least 13 occasions. Excellent linearity was obtained over this range, with good precision and accuracy (Supplemental Table 10).

SUPPLEMENTAL TABLE 10

Inter-day accuracy and precision of piperaquine assay

Nominal concentration (ng/mL)	Mean (ng/mL)	SD (ng/mL)	CV (%)	*n*	Accuracy (%)
0.25	0.247	0.03	11.9	28	98.5
0.5	0.496	0.04	7.6	20	99.2
1.0	1.06	0.07	6.6	23	106.0
5.0	5.06	0.21	4.1	25	101.3
10	10.2	0.43	4.3	25	101.7
50	49.3	2.1	4.2	24	98.7
100	99.2	3.0	3.0	23	99.2
250	244.6	12.0	4.9	25	97.9
500	496.1	18.8	3.8	25	99.2
1,000	1,023.0	23.8	2.3	13	102.3

CV = coefficient of variation.

Piperaquine intraday precision

Calibration standards of plasma PPQ concentrations from 0.25 to 500 ng/mL were analyzed on the same day (Supplemental Table 11). Excellent linearity was obtained over this range, with good precision and accuracy.

SUPPLEMENTAL TABLE 11

Intraday accuracy and precision of piperaquine assay

Nominal concentration (ng/mL)	Mean (ng/mL)	SD (ng/mL)	CV (%)	*n*	Accuracy (%)
0.25	0.252	0.040	15.97	5	100.4
0.5	0.530	0.034	6.40	5	106.0
1.0	1.048	0.067	6.35	5	104.8
5.0	5.116	0.170	3.32	5	102.3
10	10.494	0.541	5.15	5	104.9
50	49.216	1.365	2.77	5	98.4
100	101.335	1.603	1.58	5	101.3
250	252.820	8.658	3.42	5	101.1
500	502.052	30.969	6.17	5	100.4

CV = coefficient of variation.

- The LLOQ using 50 µL of plasma was 0.25 ng/mL, with a CV of 11.9% and an accuracy of 98.5%.
- A quadratic regression equation $y = ax^2 + bx + c$, where x is the amount of drug and y is the peak area ratio, was used to determine the concentrations of unknowns and QC samples. A typical regression equation of a calibration curve was as follows:
- $a = 2.15618 \times 10^{-5}$, $b = 0.12777$, $c = 0.02025$, with $r = 0.99780$ and relative standard deviation (RSD) of 0.02716.

Quality control plasma samples.

- Paired QC samples for low, mid-, and high concentrations were included with each analytical batch, and batches that had QC failures as per DE (Pharm) other SOP002—HPLC method validation and quality assurance were submitted for reassay. Results of accuracy and precision of QC samples (low–mid–high concentrations) that fell within acceptance criteria are shown for PPQ in Supplemental Table 12.

SUPPLEMENTAL TABLE 12

Accuracy and precision of piperaquine of QC samples

Nominal concentration (ng/mL)	1	100	400
No. of observations	41	44	45
Mean (ng/mL)	0.973	97.3	389
SD (ng/mL)	0.08	4.24	23.9
CV (%)	8.0	4.4	6.2%
Accuracy (%)	97.3%	97.3%	97.2%

CV = coefficient of variation; QC = quality control.

SUPPLEMENTAL MATERIAL 6

SUPPLEMENTAL TABLE 13

Plasma concentrations of PPQ and DAQ after 3-day regimens of Artequick and Coarsucam in healthy Vietnamese volunteers

3-day drug regimen	Drug	Dose	Time (hours) after dose	Concentration median (IQR) (ng/mL)	
Artequick	PPQ	1st	3	655.8	422.1–848.5
Artequick	PPQ	2nd	3	879.7	605.1–1,233.3
Artequick	PPQ	3rd	3	975.7	693.9–1,264.7
Artequick	PPQ	1st	24	125.1	101.9–149.5
Artequick	PPQ	2nd	24	308.7	198.8–390.6
Artequick	PPQ	3rd	24	256.0	234.4–338.8
Coarsucam	AQ	1st	3	15.3	11.6–21.3
Coarsucam	AQ	2nd	3	20.2	16.2–25.7
Coarsucam	AQ	3rd	3	20.6	17.5–25.0
Coarsucam	DAQ	1st	3	216.4	187.3–290.1
Coarsucam	DAQ	2nd	3	366.4	318.7–524.9
Coarsucam	DAQ	3rd	3	555.9	447.4–637.0
Coarsucam	DAQ	1st	24	77.3	65.4–94.4
Coarsucam	DAQ	2nd	24	169.4	148.6–198.2
Coarsucam	DAQ	3rd	24	154.6	143.2–185.9

AQ = amodiaquine; DAQ = desethylamodiaquine; IQR = interquartile range; PPQ = piperaquine.