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Journal of Chromatography B, 774 (2002) 195–203

JOURNAL OF CHROMATOGRAPHY B

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Development and validation of a high-performance liquid chromatography–mass spectrometry assay for the determination of artemether and its metabolite dihydroartemisinin in human plasma

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Received 27 December 2001; received in revised form 14 March 2002; accepted 9 April 2002

Abstract

A sensitive and selective method is described for the determination of artemether and its active dihydroartemisinin metabolite in human plasma using artemisinin as internal standard. The method consists of a liquid–liquid extraction with subsequent evaporation of the supernatant to dryness followed by the analysis of the reconstituted sample by liquid chromatography–mass spectrometry (LC–MS) in single ion monitoring mode using atmospheric pressure chemical ionization (APCI) as an interface. Chromatography was performed on a C_{18} reversed-phase column using acetonitrile–glacial acetic acid 0.1% (66:34) as a mobile phase. The method was fully validated over a concentration range of 5–200 ng/ml using 0.5 ml of human plasma per assay. Stability assessment was also included. The method was applied to the quantification of artemether and its metabolite in human plasma of healthy volunteers participating in pharmacokinetic drug–drug interaction studies. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Artemether; Dihydroartemisin

1. Introduction

Co-artemether is an oral fixed-dose combination tablet of artemether (a derivative of artemisinin) and lumefantrine, an antimalarial synthesized and developed by the Academy of Military Medical Sciences in Beijing, People's Republic of China. This combination was registered in China in 1992 for the treatment of *Plasmodium falciparum* malaria, and has been subsequently further developed by Novartis Pharmaceuticals (Coartem®/Riamet®).

The pharmacokinetics of artemether (A) and its active main metabolite dihydroartemisinin (DHA) have previously been characterized using a high-performance liquid chromatography with electrochemical detection in the reductive mode [1]. However, this analytical method is difficult to use routinely due to the very rigorous conditions that should be applied to prevent dissolved oxygen from entering the flowcell. Therefore, the development of a new, easier to use and at least equally sensitive method turned out to be necessary. A combined HPLC–mass spectrometric method (LC–MS) was found to be the favorite choice.

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PII: S1570-0232(02)00207-6

In the literature, LC–MS using electrospray ionization detection are described for the determination of artemisinin [2] and for A and DHA [3] but none of them were validated in plasma. A thermospray mass spectrometric method [4] is also described for the determination of DHA (as a metabolite of Arteether) in human plasma but was used only for metabolite identification. More recently a gas chromatography–mass spectrometric method [5] for the determination of A and DHA in human plasma is described.

The electrospray ionization detection was first applied to A and DHA but no predominant ion with enough intensity was found for these two compounds. Therefore, a more intensive ionization was tested, and atmospheric pressure chemical ionization (APCI) was selected.

The present paper describes a sensitive and selective analytical method that meets the accepted criteria for bioanalytical method validation [6].

2. Experimental

2.1. Solvents and chemicals

Artemether (A), $C_{16}H_{26}O_5$, mol. wt.=298.38 and dihydroartemisinin (DHA), $C_{15}H_{24}O_5$, mol. wt.= 284.35 were synthesised by Novartis Pharma AG, Basle, Switzerland. DHA is a mixture of α and β tautomers with unknown ratio of α versus β. Artemisinin was also provided by Novartis Pharma AG and used as internal standard (I.S.). The chemical structures of A and DHA are shown in Fig. 1.

All solvents and reagents were of analytical grade and used without further purification. Ethyl alcohol and acetonitrile were obtained from Carlo-Erba (Milan, Italy). 1-Chlorobutane, isooctane, glacial acetic acid and saturated sodium chloride were obtained from Merck (Darmstadt, Germany). Water was deionized, filtered and purified on a Milli-Q Reagent Grade Water System from Millipore (St Quentin en Yvelines, France).

Drug-free human plasma was obtained from ''Les Etablissements Français du Sang'' (Bois-Guillaume, France) where blood was collected from volunteers in tubes containing citrate–phosphate–dextrose or heparin. After centrifugation, the plasma was transferred into polypropylene tubes and stored at or below −18 °C.

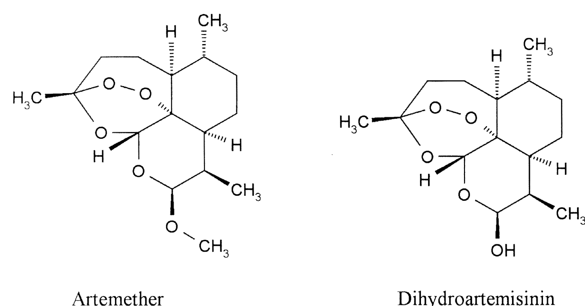

Fig. 1. Chemical structure of Artemether and its DHA metabolite.

2.2. Instrumentation

The HPLC system consisted of a 1100 pump and a 1050 autosampler (Hewlett-Packard-Agilent, Arnhem, The Netherlands). Masses were acquired on a TSQ API II spectrometer equipped with an atmospheric pressure chemical ionisation (APCI) interface (Thermo-Finnigan, San Jose, CA, USA) and connected to a PC running the standard software Xcalibur (version 1.1).

2.3. Liquid chromatography–mass spectrometry

HPLC was carried isocratically at room temperature using an Alltima C_{18} 5 μm column (150×4.6 mm, Alltech, Deerfield, USA) fitted with a guard column of the same packing materiel (7.5×4.6 mm). The mobile phase consisted of acetonitrile–0.1% glacial acetic acid (66:34, v/v). The flow-rate was 1 ml/min. The duration of the analytical time was 14 min followed by a 15-min flush step at 2 ml/min with acetonitrile (the flush was not always found necessary and its duration depends on the spectrometer used).

The mass spectrometer was operated in positive ion mode, single ion monitoring with a manifold temperature set at 70 °C, the dynode at 15 kV and the electron multiplier at 1400 V. Nitrogen was used as nebulizer gas at a pressure of 586 kPa with a vaporizer temperature at 400 °C. The capillary tem-

perature was set at 300 °C and the corona needle discharge at 5 μA. Nitrogen was also used as auxiliary gas at 15 units on the flow meter.

2.4. Preparation of calibration standards and quality control samples

Primary stock solutions were prepared by dissolving the compounds or internal standard in ethyl alcohol. Appropriate dilutions of the stock solutions with water–ethyl alcohol (50: 50), v/v) were made subsequently in order to prepare the working solutions in the range 50–2000 ng/ml for both compounds. Two different series of stock solutions were prepared from different weightings for calibration standards (Cs) and quality control samples (QCs). The I.S. working solution is used at a concentration of 2000 ng/ml. All the solutions were prepared in polypropylene flasks and stored in the darkness between 2 and 8 °C. Cs and QCs in the concentration range of 5–200 ng/ml were prepared for calibration, accuracy and precision, quality control and stability assessment by spiking 0.5 ml of drug-free human plasma with appropriate volume of working solutions. The samples were prepared as described below (Section 2.5).

2.5. Sample preparation

Appropriate volume of I.S. working solution was added to Cs, QCs or actual samples following by 0.125 ml of NaCl saturated solution and 2.5 ml of 1-chlorobutane–isooctane (55:45, v/v) in a silanized glass tube. The tubes were placed on a horizontal shaker for 5 min at a velocity of 250 rpm. After a 15-min centrifugation at 3650 *g* at 15 °C, the organic layer was transferred into another silanized conical tube. The solvent was evaporated to dryness at 30 °C under a nitrogen stream. The residue was dissolved in 100 μl of ethanol–0.1% glacial acetic acid (50:50, v/v) by vortex mixing and the solution was transferred into a conical polypropylene insert inside amber glass microvials.

All liquid transfers were done manually using RAININ-Gilson electronic pipettors (Villiers-le-Bel, France).

2.6. Validation

2.6.1. Selectivity

Six human plasma from six individual healthy donors receiving no medication were extracted and analysed for the assessment of potential interferences with endogenous substances. The apparent response at the retention times of A, DHA and I.S. was compared to the response at the lower limit of quantitation (LLOQ) for A and DHA and to the response at the working concentration for I.S.

2.6.2. Recovery

The recovery of A and DHA was evaluated in duplicate at three concentration levels (10, 40 and 200 ng/ml) from the peak area of assayed sample by comparison to the one of an assayed drug-free sample spiked with compounds after their processing.

2.6.3. Calibration and sample quantification

Calibration standards at levels of 5, 10, 20, 50, 100, 200 ng/ml ($n=2$, at each level) were extracted and assayed as described above, on three different days. Calibration curves ($y = ax + b$), represented by the plots of the peak-area ratios (y) of A or DHA to I.S. versus the concentration (x) of the calibration standards, were generated using weighted ($1/x^2$) linear least-squares regression as the mathematical model. Actual, quality control and stability samples were calculated from the resulting area ratio of A or DHA (only the α-tautomer was considered) and the regression equation of the calibration curve.

2.6.4. Accuracy and precision

Intra-day accuracy and precision were evaluated by analysis of QCs at levels of 5, 8, 40 and 160 ng/ml ($n=6$ at each level) on the same day. These levels were chosen to demonstrate the performance of the method and to determine the lower limit of quantitation (LLOQ) of the method. The upper limit of quantitation (ULOQ) was given by the highest level of the calibration curve. Samples with concentrations above this ULOQ should be diluted prior to re-analysis. To assess the inter-day accuracy and precision, the intra-day assays were repeated on three different days. The overall performance was calculated.

2.6.5. Stability

The effect of three freeze–thaw cycles, the compound stability at room temperature in human plasma and in working solutions under daylight and in darkness, were evaluated by repeated analysis ($n=3$) of stability control samples spiked with working solution at low and high concentrations. The stability was expressed as a percentage of the theoretical value. Long-term stability was also tested after storage at or below −70 °C on spiked and clinical samples. Stability was expressed as a percentage of the initial value.

3. Results

3.1. Mass spectra analysis

The full scan mass spectra of A and DHA after direct injection in mobile phase are presented in Figs. 2 and 3. Protonated molecules $(MH)^+$ of A and DHA were not detected. The predominant protonated molecules found were (MH^+–CH_3OH) m/z 267 and (MH^+–CH_3OH–C_2H_5OH) m/z 221 for A and (MH^+–OH) m/z 267 and (MH^+–OH–C_2H_5OH) m/z 221 for DHA. The protonated molecule was detected for I.S. at m/z 283. The mass spectrometric parameters were optimised to obtain the higher signal for the selected ions 221 or 267. The method was fully validated using ion 267 for A and DHA. A within-day validation was also performed monitoring ion 221 for both compounds with satisfactory results.

3.2. Retention times and selectivity

Observed retention times were about 10.4, 3.6, and 5.1 min for A, DHA (α) and I.S., respectively (Fig. 4). The α and β tautomers of DHA were separated (β retention time 4.6 min). Only the α tautomer (the predominant peak) of DHA was taken into account for quantitation. Under the chromatographic conditions, the ratio α versus β remained constant at around 3. No additional peak due to endogenous substances that could have interfered with the detection of the compounds of interest was observed. Representative chromatograms for A, DHA and I.S. in actual plasma sample are presented in Fig. 4.

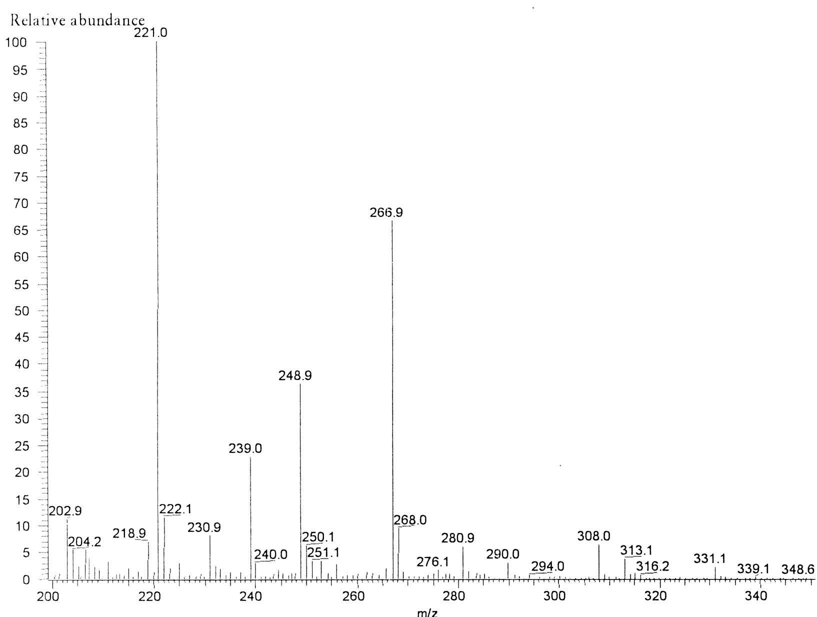

Fig. 2. Full scan mass spectra of Artemether.

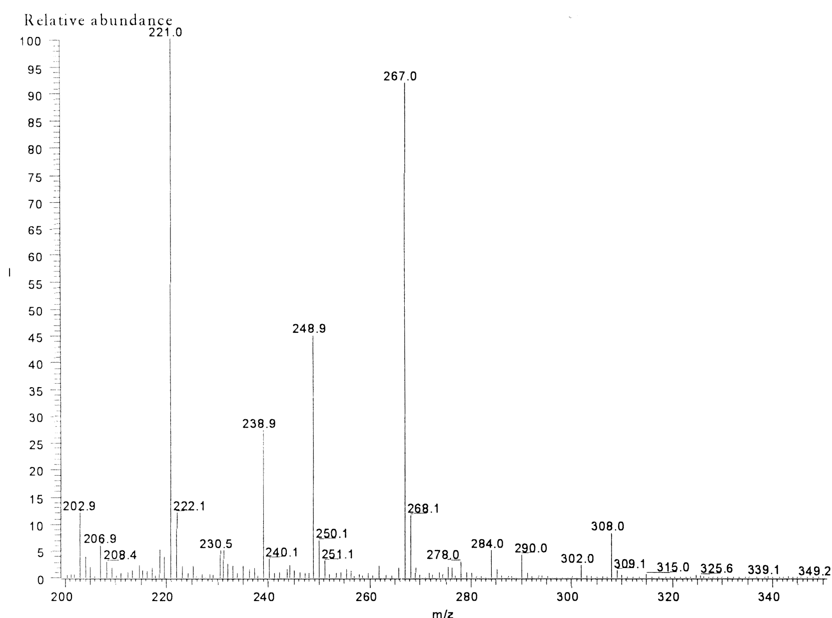

Fig. 3. Full scan mass spectra of Dihydroartemisinin.

Maximum interfering peaks amounted to 9.6, 13.2 and 1.1% for A, DHA and I.S., respectively.

3.3. Recovery

The mean recovery after liquid–liquid extraction with 1-chlorobutane–isooctane (55:45, v/v) was 76% (range 72–77%) and 84% (range 67–109%) for A and DHA, respectively. These results suggested that there was no relevant difference in extraction recovery at different concentration levels for both A and DHA. I.S. recovery was also tested and was 94% at the working concentration of 40 ng/ml.

3.4. Linearity

Linear calibration curves were obtained with a coefficient of correlation (r) usually higher than 0.995. Inter-day repeatability on 3 different days is shown in Table 1.

For each calibration standard level, the concentration was back calculated from the linear regression curve equation. Inter-day variability is presented in Table 2. The precision was $\leq$10% and mean accuracies were within 5% of the nominal values for both compounds.

3.5. Precision and accuracy

The LLOQs were defined as the lowest drug concentration, which can be determined with an accuracy of 80–120% and a precision $\leq$20% on a day-to-day basis [6].

The results (Table 3) satisfactorily met the acceptance criteria: mean accuracy within 85–115% and C.V.$\leq$15% (80–120% and $\leq$20% at LLOQ). LLOQs were set at 5 ng/ml for both compounds.

3.6. Stability

A and DHA were stable for at least 8 months in both actual and spiked human plasma samples when frozen at or below −70 °C. The mean (±SD) recoveries (from the first determination), for actual samples, were 104±18% ($n=17$) and 84±15% ($n=12$) for A and DHA, respectively. They were 86 and 114% for A and DHA respectively, in spiked sam-

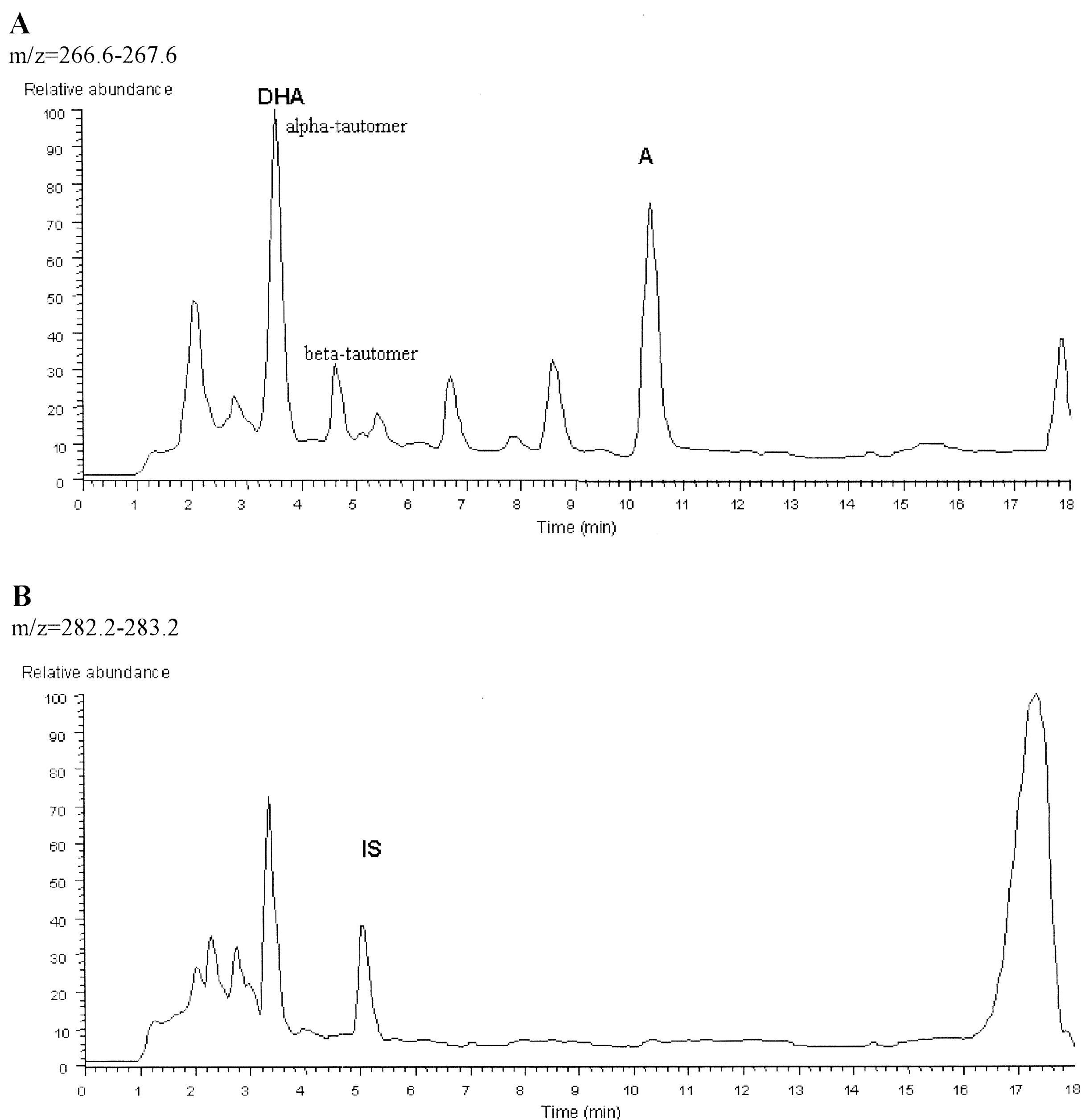

Fig. 4. Representative chromatograms obtained following extraction of Artemether (113 ng/ml), its DHA metabolite (123 ng/ml) and the internal standard (20 ng/ml) from actual human plasma sample.

ples at 8 ng/ml concentration, and 101 and 109% respectively at 160 ng/ml.

A and DHA were stable for at least 6 h at room temperature in spiked human plasma samples; the mean recoveries from the nominal concentration were 98 and 86% for A and DHA, respectively at 20 ng/ml concentration, and 101 and 89%, respectively at 100 ng/ml.

Table 1
Assay linearity of the method

Date	Slope	y-Intercept	Coefficient of correlation
Artemether			
Mean	0.01054490	0.00165010	0.9965
SD	0.00055165	0.00419780	0.0010
Dihydroartemisinin			
Mean	0.00875723	−0.02107860	0.9966
SD	0.00009344	0.00756640	0.0011

A and DHA in working solutions were found to be stable for at least 7 weeks at +2–8 °C; the mean recoveries ($n=3$) from the nominal concentrations were 101 and 88% for A and DHA, respectively at 20 ng/ml concentration, and 107 and 96%, respectively at 100 ng/ml. They were also found to be stable in working solutions for at least 6 h at room temperature in darkness; the mean recoveries ($n=3$) from the nominal concentrations were 117 and 87% for A and DHA, respectively at 20 ng/ml concentration, and 120 and 101%, respectively at 100 ng/ml.

Under daylight at room temperature, A and DHA were found to be stable for at least 6 h at 100 ng/ml concentration (mean recoveries ($n=3$), 120 and 110% for A and DHA respectively) but not at 20 ng/ml (mean recoveries ($n=3$), 78 and 69% for A and DHA respectively).

Extracts at concentrations of 8 and 100 ng/ml were found to be stable on the autosampler at room temperature for at least 14 h.

Artemether mean recovery values after three freeze–thaw cycles were 89 and 95% of the nominal value for 20 and 100 ng/ml concentrations, respectively, whereas DHA mean recovery values were 88 and 87%, respectively.

3.7. Application to clinical sample analysis

The method monitoring ion 267 was applied to a pharmacokinetic interaction study of the combined (sequential) administration of oral co-artemether and i.v. quinine (Fig. 5). The plasma samples were collected from 28 healthy volunteers. Concentrations of A and DHA were determined in about 500 samples. Within this assay, batch runs of about 55 injections each were performed which included clinical samples, 10 Cs, 6 QCs and drug-free samples. The within-assay accuracy data of QCs were in the range 95–113% and 99–100% of the nominal value for A and DHA, respectively, with coefficients of variation of 9–17% and 8–16%, respectively.

The method monitoring ion 221 was applied to another pharmacokinetic interaction study of the combined single dose administration of oral co-artemether and ketoconazole. The study was initially monitoring with ion 267 but a chromatographic peak interfering with A in the 5 first tested subjects prevented monitoring this ion. The plasma samples were collected from 16 healthy volunteers. Concentrations of A and DHA were determined in about 500 samples. Within this assay, batch runs of about 55 injections each were performed which included

Table 2
Back-calculated concentrations from calibration curves

Nominal conc. [ng/ml]	5.00	10.0	20.0	50.0	100	200
Artemether						
Mean accuracy [%]	97	105	102	100	99	98
Precision [%]	10	6	7	4	6	4
N	5	6	6	6	6	6
Dihydroartemisinin						
Mean accuracy [%]	99	105	99	101	99	100
Precision [%]	7	10	7	5	4	4
N	6	4	6	6	6	6

Accuracy: 100% measured concentration/nominal concentration.
Precision: Coefficient of variation (100% standard deviation/mean).
N: Number of determinations.

Table 3
Assessment of the accuracy and precision of the method

Nominal conc. [ng/ml]	5.00	8.00	40.0	160
Artemether				
Intra-day accuracy [%] (day 1)	95	89	90	98
Intra-day precision [%] (day 1)	3	11	4	2
Intra-day accuracy [%] (day 2)	90	91	88	93
Intra-day precision [%] (day 2)	12	7	10	7
Intra-day accuracy [%] (day 3)	105	97	96	97
Intra-day precision [%] (day 3)	15	7	6	3
Overall accuracy [%]	97	92	91	96
Overall precision [%]	13	9	7	5
Number of determinations	17	18	18	18
Dihydroartemisinin				
Intra-day accuracy [%] (day 1)	97	92	90	93
Intra-day precision [%] (day 1)	6	9	5	6
Intra-day accuracy [%] (day 2)	99	95	104	91
Intra-day precision [%] (day 2)	12	6	8	3
Intra-day accuracy [%] (day 3)	104	103	93	95
Intra-day precision [%] (day 3)	14	9	7	4
Overall accuracy [%]	100	96	95	93
Overall precision [%]	11	9	9	5
Number of determinations	17	18	17	18

Accuracy: 100% measured concentration/nominal concentration.
Precision: Coefficient of variation (100% standard deviation/mean).

clinical samples, 10 Cs, 6 QCs and drug-free samples. The within-assay accuracy data of QCs were in the range 97–102% and 95% of the nominal value for A and DHA, respectively, with coefficients of variation of 10–12% and 9–16%, respectively.

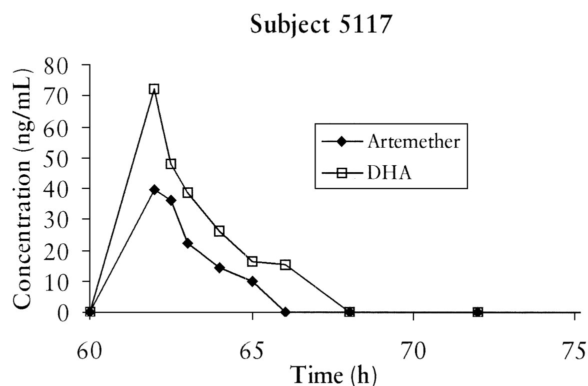

Fig. 5. Plasma concentrations of artemether and DHA following administration of the last (sixth) dose of co-artemether in combination with a subsequent 2 h i.v. infusion of quinine.

4. Conclusion

The LC–MS method reported in this paper was validated according to internationally accepted criteria [6]. APCI technique has proven effective in generating ions closed to the protonated molecule with sufficient intensity to be monitoring quantitatively, accurately and selectively.

The method consists of sample preparation by liquid–liquid extraction, followed by chromatographic separation on a C_{18} column and detected in the SIM mode. The method is sensitive enough for the determination of artemether and its active metabolite DHA in human plasma for pharmacokinetic analyses and was validated in the range 5–200 ng/ml using 0.5 ml of human plasma per assay. The method was

applied successfully to the analysis of over 1000 samples generated during clinical trials.

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