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Short communication

A liquid–liquid LC/MS/MS assay for the determination of artemether and DHA in malaria patient samples

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ABSTRACT

A solvent extraction method was developed and validated for the determination of the antimalarial drug, artemether and its active metabolite dihydroartemisinin (DHA) in malaria patient plasma samples. An AB Sciex 4000 triple quadrupole mass spectrometer in the multiple reaction monitoring (MRM) mode was used for detection in the positive ionisation mode. Liquid–liquid extraction was followed by PFP liquid chromatography and tandem mass spectrometry. Stable isotope labelled artemether and DHA was used as internal standards. The calibration range was between 2.00 and 500 ng/ml for both artemether and DHA during the original validation and the upper limit was lowered to 200 ng/ml during a re-instatement validation, prior to sample analysis. The assay was used to measure artemether and DHA in human plasma samples, which were generated from a safety and efficacy clinical trial in Mbarara, Uganda; as well as for a pharmacokinetic interaction study between the antimalarial combination artemether/lumefantrine and combination antiretroviral therapy including nevirapine in HIV-infected adults.

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1. Introduction

Malaria is caused by *Plasmodium* protozoan parasites and is transmitted to humans by female *Anopheles* mosquitoes. It is a major threat to the human race and results in more than a million deaths per year and around 250 million cases annually. Three billion people are at risk of infection in 109 malarious countries [1].

Artemether is a semi-synthetic derivative of artemisinin, and artemisinin was first isolated as an active antimalarial from *Artemisia annua* by Chinese scientists in 1971 [2]. A combination tablet of lumefantrine and artemether is now available from Novartis as Coartemether (Riamet®, Coartem®) [3].

Liquid–liquid [4–8], solid phase [10] and protein precipitation [11] extraction methodologies have been previously applied in LC/MS/MS methods for the determination of artemether and DHA in plasma samples. The liquid–liquid extraction methods were used for healthy human [4–6] and rat [7,8] PK studies. The solid phase extraction method was used for a malaria patient study [10] and the protein precipitation method was used for a healthy human PK study [11].

Lindegardh and co-authors published a paper in 2008 where they discuss major problems with an assay that was developed, according to FDA guidelines, for the analysis of artesunate and its metabolite dihydroartemisinin (DHA) in malaria patient plasma using protein precipitation and liquid chromatography coupled to positive tandem mass spectroscopy [9,12]. Variable degradation of the artemisinins was observed in patient samples from clinical pharmacokinetic malaria studies. They also observed that haemolytic products related to sample collection and malaria infection degraded the compounds when using their protein precipitation method. They argued that the addition of organic solvents during sample processing caused analyte and metabolite degradation. Their solution was to develop a solid phase extraction method on μ-elution Oasis HLB columns in 96-well format. This method performed well during patient sample analysis and is an excellent option for the determination of artemether and DHA, but the extraction method is relatively expensive.

The observation of artemether and DHA degradation due to exposure of malaria patient samples to organic solvents during sample processing, including addition of low volumes of internal standard in an organic solvent, raises concerns that similar degradation may occur in malaria patient samples exposed to organic solvents during liquid–liquid extraction. A number of assays have been successfully validated for artemether and DHA using liquid–liquid extraction as part of the assay [4–8]. These assays have been applied in healthy volunteer and animal studies but their suitability for use in malaria patient studies has not been evaluated.

We describe here a robust method, employing liquid–liquid extraction with an organic solvent, for the determination of

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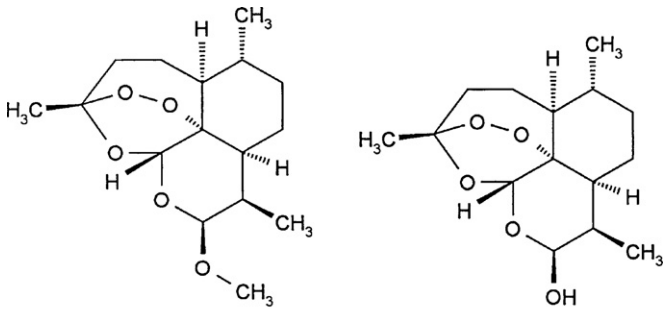

Fig. 1. Chemical structure of artemether and DHA.

artemether and DHA in plasma. The method was found suitable for the determination of these analytes in samples from malaria patients and in haemolysed samples.

2. Experimental

2.1. Materials and chemicals

Methanol (LiChrosolv®), acetonitrile (LiChrosolv®), formic acid (pro analysis), acetic acid (suprapur®) and water (LiChrosolv®) were purchased from Merck kGaA, Darmstadt, Germany. The artemether and DHA reference standards and isotope-labelled internal standards were supplied by Novartis Pharma AG (Basel, Switzerland). Drug free plasma was obtained from the Blood Bank at the Groote Schuur Hospital, South Africa. A Phenomenex Luna, PFP(2) 100A, 50 mm × 2.0 mm column (Phenomenex, USA) was used for retaining artemether, DHA and the isotope-labelled internal standards.

2.2. Chemical structures

Chemical structures of artemether and DHA are presented in Fig. 1.

2.3. Instrumentation

The mobile phase was delivered with an Agilent 1200 series binary pump and the samples injected with an Agilent 1200 series autosampler (Agilent, CA, USA). Detection was performed by an AB Sciex API 4000 mass spectrometer (AB Sciex, Ontario, Canada) fitted with a Turbo V™ ion source.

2.4. Preparation of calibration standards and quality control standards

The standard and quality control preparation procedure was performed on ice. Calibration standards were prepared in blank human plasma. Two sets (one for artemether and one for the metabolite) of stock solutions (SS) were prepared in ethanol at 1000 μg/ml (SS1), 100 μg/ml (SS2), 10 μg/ml (SS3) and 1 μg/ml (SS4). Blank plasma (5 ml each) was spiked with these stock solutions (analyte and metabolite) to attain the desired calibration standards (2.00, 8.00, 20.0, 50.0, 100, 180, 320 and 500 ng/ml). The same methodology was used for the preparation of quality controls (2.00, 6.00, 200 and 400 ng/ml). The calibration standards and quality control standards were briefly vortexed, aliquotted into polypropylene tubes and stored at approximately −70 °C.

2.5. Extraction procedure

The extraction procedure was performed on ice and in polypropylene test tubes. The plasma samples were thawed on ice and briefly vortexed. The samples (100 μl) were pipetted into polypropylene tubes. Normal blank plasma (100 μl), which contained both stable isotope labelled internal standards (ISTD) at 100 ng/ml, and 200 μl of a Britton Robinson universal buffer (0.1 M, pH 10) was added. Ethyl acetate (2 ml) was added as the organic solvent, the samples were vortexed for 1 min and centrifuged for 5 min at 16,000 rcf. The organic phase (1.6 ml) was transferred to clean polypropylene tubes and evaporated under vacuum in a rotor evaporation system at 30 °C for 1.5 h. Mobile phase (100 μl) which consisted of methanol and ammonium acetate (10 mM) with 0.1% acetic acid (65:35, v/v) was added to the dry samples. The samples were vortexed for 30 s and transferred to 96 well polypropylene plates. Ten microlitres was injected onto the HPLC column.

2.6. Mass spectrometry

Electrospray ionisation (ESI) was performed in the positive ion mode with nitrogen as the nebulizing, turbo spray and curtain gas with the optimum values set at 50, 60 and 20 psi, respectively. The heated nebulizer temperature was set at 300 °C. The ionspray voltage was set at 5000 V. The instrument response was optimised for artemether by infusing a solution of the drug dissolved in mobile phase at a constant flow. The same methodology was used to optimise the response of the instrument for the metabolite (DHA) and the 2 stable isotope labelled internal standards. The pause time was set at 5 ms and the dwell time at 150 ms. The collision gas (N_2) was set at 5 (arbitrary values).

The AB Sciex API 4000 mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the ammonium adduct ions at *m*/*z* 316.2 to the product ions at *m*/*z* 163.1 for artemether, the ammonium adduct ions at *m*/*z* 302.2 to the product ions at *m*/*z* 163.0 for DHA, the ammonium adduct ions at *m*/*z* 320.1 to the product ions *m*/*z* 163.1 for the isotope labelled artemether internal standard, and the ammonium adduct ions at *m*/*z* 307.2 to the product ions *m*/*z* 168.2 for the isotope labelled DHA internal standard. The instrument was interfaced with a computer running Analyst version 1.4.2 software.

2.7. Liquid chromatography

Chromatography was performed on a Phenomenex Luna, PFP (50 mm × 2.0 mm, 5 μm) analytical column. The mobile phase consisted of methanol and ammonium acetate (10 mM) with 0.1% acetic acid (65:35, v/v) and was delivered with a gradient (65–90% methanol over 2.9 min, kept at 90% for another 2.1 min, brought back to 65% in 0.1 min, kept at 65% methanol for another 2.9 min) at a flow rate of 0.5 ml/min for 8 min. The analytical column was kept in a column compartment at a constant temperature of 35 °C. An Agilent 1200 series autosampler injected 10 μl onto the HPLC column. The injection needle was rinsed with mobile phase (methanol and ammonium acetate (10 mM) with 0.1% acetic acid (65:35, v/v)) for 10 s using the flush port wash station. The samples were cooled to 5 °C while awaiting injection. Representative raw chromatograms at the limit of quantification (LOQ) for artemether and DHA are presented in Figs. 2 and 3, respectively.

2.8. Method validation

2.8.1. Calibration standards and quality controls

The calibration curves for artemether and DHA were validated by analysing plasma quality control samples in six fold at high,

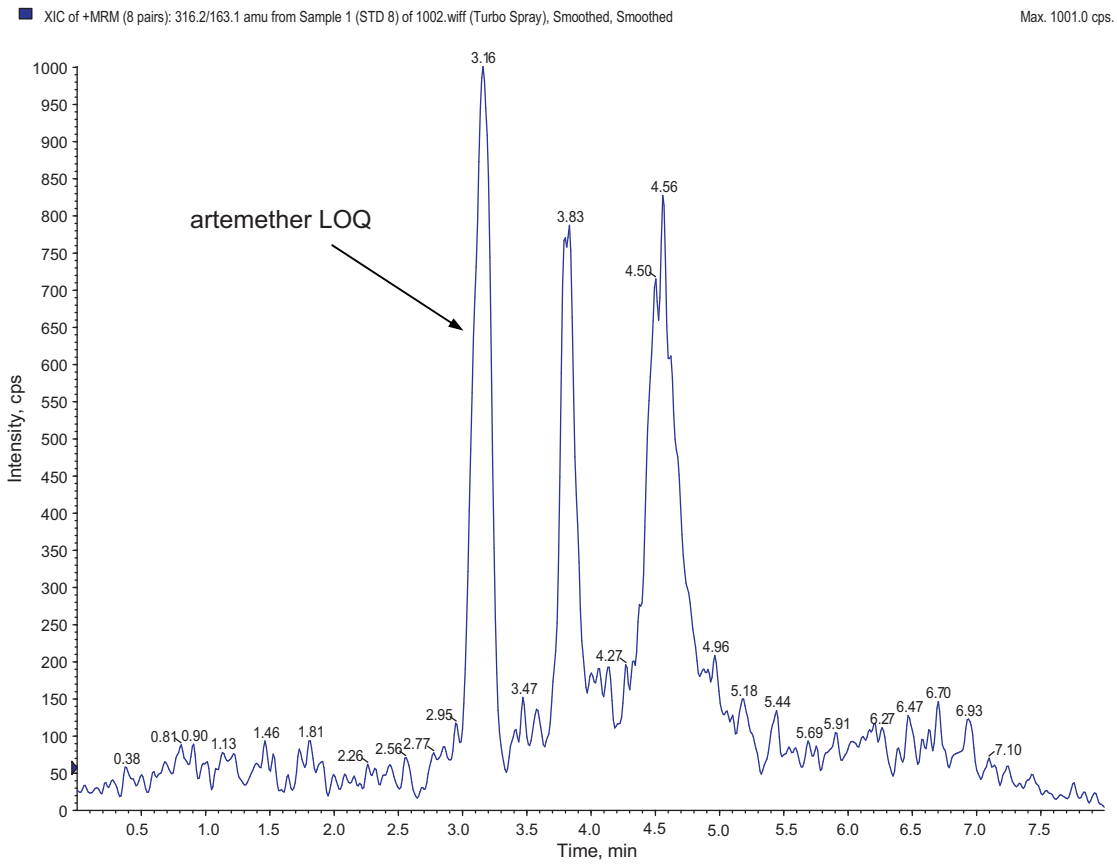

Fig. 2. Chromatogram of an artemether calibration standard at LOQ (2.00 ng/ml).

medium, low and LOQ concentrations (400, 200, 6 an 2 ng/ml, respectively) over a period of 3 days to determine the intra- and inter-day accuracy and precision. The quality control values were interpolated from calibration curves containing eight different concentrations spanning the concentration range of 2–500 ng/ml for both artemether and DHA. Calibration graphs were constructed for artemether and DHA using a quadratic regressions of the drug/metabolite peak-area ratios of the analyte/metabolite to the ISTD vs. nominal drug concentrations. A reinstatement validation with a reduced calibration range (2–200 ng/ml for both artemether and DHA) was performed prior to sample analysis.

2.8.2. Recovery

Recoveries of artemether and DHA were initially evaluated during the method development phase of the project. Different extraction solvents (low to high polarities) and pH conditions (between 3 and 11) were evaluated. Consistent recoveries were obtained with ethyl acetate at pH 10. Recoveries of artemether and DHA were formally evaluated at relatively low and high concentrations during the validation phase of the project. Absolute recoveries of the analyte and metabolite were determined in five fold by extracting blank plasma samples spiked with artemether and DHA at appropriate concentrations. Recoveries were calculated by comparison of the analyte/metabolite peak-areas of the extracted samples with reference samples prepared in mobile phase with background extract components present.

2.8.3. Stock solution stability

Stock solutions of artemether and DHA were prepared in ethanol. The test sample was left at room temperature for 2 h and the reference sample was kept at −70 °C. Both the reference and test samples were diluted with mobile phase at a concentration of 500 ng/ml (both artemether and DHA) and were analysed with the other stability samples.

2.8.4. Freeze and thaw stability

In order to ascertain freeze–thaw stability, low (20 ng/ml) and high (320 ng/ml) standards were frozen at −70 °C, and put through three freeze and thaw cycles (on ice) and were analysed against a valid calibration curve.

2.8.5. Benchtop stability

In order to ascertain benchtop stability, low (20 ng/ml) and high (320 ng/ml) standards were frozen at −70 °C, and left on the bench on ice for 2 h and were analysed against a valid calibration curve.

2.8.6. On-instrument stability

A 24 h on-instrument stability evaluation of artemether, DHA and the isotope-labelled internal standards was performed. Six high and 6 low quality controls were extracted and analysed over 2 days. The samples were extracted and analysed on day 1, left on the autosampler for 24 h and analysed again.

2.8.7. Long term matrix stability

Five high, medium and low quality control standards were stored at −70 °C and will be analysed against newly prepared calibration standards in about 6 months to determine long term matrix stability.

2.8.8. Matrix effect evaluation

The matrix effect evaluation publication by Matuszewski et al. was followed to evaluate the influence of matrix background components to analyte, metabolite and internal standards ionisation [13].

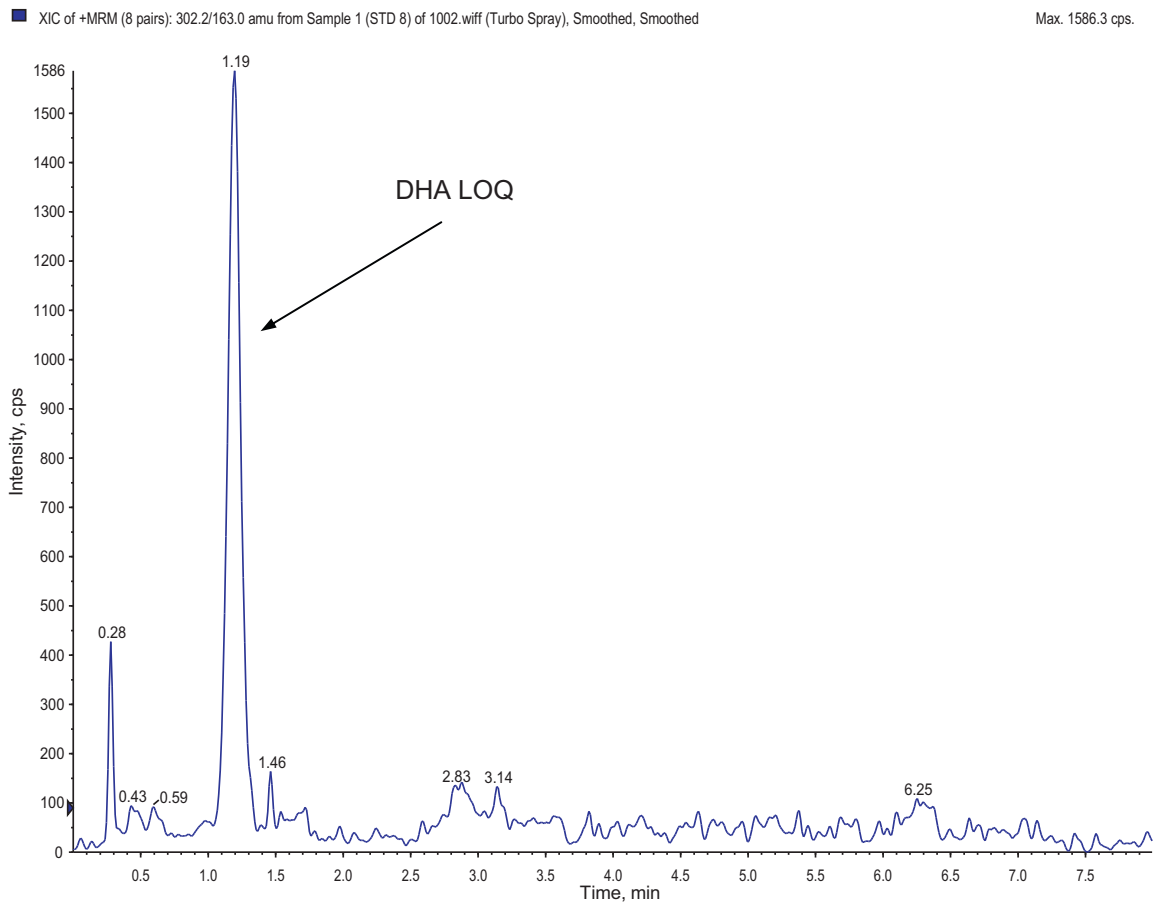

Fig. 3. Chromatogram of a DHA calibration standard at LOQ (2.00 ng/ml).

The matrix effect was evaluated by extracting 10 different matrix samples for the relatively high and low experiments in duplicate. The dried samples were reconstituted with mobile phase which was spiked with artemether, DHA, deuterated artemether and deuterated DHA at 400 ng/ml (relatively high) and at 40 ng/ml (relatively low), respectively.

2.8.9. Haemolysis evaluation

Haemolysed plasma samples were prepared at 1% and 2% and were evaluated at relatively high (240 ng/ml) and low (12 ng/ml) concentrations for artemether and DHA. The measured concentrations of the test samples were determined and compared with the concentrations of reference plasma samples to calculate the overall accuracy.

2.8.10. Specificity and carry-over

The very high specificity of the LC/MS/MS assay procedure precludes the detection of any compounds that do not possess the capability to produce the specific parent ion followed by formation of the specific product ion produced and monitored in the mass spectrometer. A double blank sample (without analyte, metabolite and ISTD) were positioned in the injection sequence immediately after the highest calibration standard in order to assess possible carry-over effects.

3. Results and discussion

The liquid–liquid extraction method performed well during the validation phase of the project. The original range was validated between 2 and 500 ng/ml for both analyte and metabolite. Several regression types were tested and the quadratic regression ($1/x$ weighting) was found to be most suited for the specific range for artemether and the quadratic regression ($1/x^2$ weighting) was found to be most suited for the specific range for DHA. The combined accuracy and precision statistics of the quality controls ($N = 18$; high, medium, low and at limit of quantification) were between 93.4% and 104.0%, and 3.4% and 14.3%, respectively, for artemether and DHA. A reduced range of 2–200 ng/ml was revalidated during reinstatement of the assay prior to sample analysis. The combined accuracy and precision statistics of the quality controls ($N = 6$; high, medium, low and at limit of quantification) were between 93.7% and 107.5%, and 4.1% and 11.5%, respectively, for artemether and DHA.

The % recoveries for artemether ($N = 5$) at relatively low and high concentrations were 80.1 (CV% = 2.6) and 76.5 (CV% = 2.3), respectively. The % recoveries for DHA ($N = 5$) at relatively low and high concentrations were 85.5 (CV% = 3.8) and 75.9 (CV% = 0.4), respectively. Stable isotope labelled artemether and DHA internal standards were used and would extract similar to artemether and DHA.

The accuracies of the stock solutions test samples compared to the reference samples were 90.2% (CV% = 3.7, $N = 3$) and 92.6% (CV% = 2.1, $N = 3$) for artemether and DHA, respectively. Artemether and DHA showed slight instability over a period of 2 h at room temperature. However, stock solutions were kept at −70 °C until they were used for standard and quality control standard preparation.

The accuracies of the artemether freeze-thaw samples were 106.0% (CV% = 4.3, $N = 3$) and 106.1% (CV% = 4.2, $N = 3$) at 20 and 320 ng/ml, respectively. The accuracies of the DHA freeze–thaw samples were 104.0% (CV% = 5.1, $N = 3$) and 93.1% (CV% = 2.3, $N = 3$) at 20 and 320 ng/ml, respectively. The test samples were put

through 3 freeze–thaw cycles and the calculated concentrations were all within 7% compared to the nominal concentrations, which indicates that the analyte and metabolite are stable through 3 freeze–thaw cycles.

The accuracies of the artemether benchtop samples were 110.3% (CV% = 2.9, $N = 3$) and 104.4% (CV% = 2.6, $N = 3$) at 20 and 320 ng/ml, respectively. The accuracies of the DHA artemether benchtop samples were 104.8% (CV% = 1.8, $N = 3$) and 96.2% (CV% = 6.0, $N = 3$) at 20 and 320 ng/ml, respectively.

The calculated concentrations of the test samples were all within 11% compared to the nominal concentrations, which indicates that the analyte and metabolite are benchtop stable for up to 2 h on ice.

Artemether, DHA and the internal standards were also stable on instrument at 5 °C for at least 24 h.

The matrix effect samples were evaluated and the coefficient of variation of the 10 peak areas of artemether and the isotope labelled artemether internal standard at the relatively high concentration were 3.8% and 3.2% with a ratio of 1.5%; and 3.8% and 3.4% with a ratio of 2.2% at the relatively low concentration. The coefficient of variation of the 10 peak areas of DHA and the isotope labelled DHA internal standard at the relatively high concentration were 2.8% and 2.8% with a ratio of 0.7%; and 4.1% and 3.6% with a ratio of 2.0% at the relatively low concentration. The background matrix components had a minimal effect on ion formation for both artemether, DHA and the internal standards.

The accuracies of the haemolysed plasma samples ($N = 5$) were between 99.4% and 108.7% for artemether and DHA, which indicate that the accurate measuring of artemether and DHA concentrations is not compromised in haemolysed samples.

Due to the high specificity of MS/MS detection, no interfering or late eluting peaks were found when analysing blank plasma extracts from six different sources. The effect of such compounds on ionisation of the analyte, metabolite and internal standards were also investigated and no significant suppression or enhancement was observed. No carry-over was observed. The LOQ (S/N > 5), defined as that concentration of artemether and DHA which can still be determined with acceptable precision (CV% < 20) and accuracy (bias < 20%) was found to be 2 ng/ml for both analyte and metabolite.

4. Application to clinical pharmacokinetic studies

The assay performed well during sample analysis of clinical samples generated from the two clinical studies as described in the abstract of the document. The precision (total-assay coefficients of variation; CV%) for artemether and DHA during sample analysis of the pharmacokinetic interaction study were less than 8% at high (160 ng/ml), medium (80 ng/ml) and low (6 ng/ml) QC levels, and were 11.5% and 8.3% at the limit of quantification, respectively. The limit of quantification was 2 ng/ml for both artemether and DHA.

The internal standard response graphs of artemether and DHA for one of the sample batches from the malaria patient study are presented in Figs. 4 and 5, respectively. Importantly, no degradation of either of the stable isotope internal standards was observed (Figs. 4 and 5), in contrast to the significant degradation observed by Lindegardh et al. [9] who used organic solvent for protein precipitation. Our results suggest that use of a non-water miscible organic solvent for liquid–liquid extraction, avoids the degradative effects of water miscible organic solvents used for protein precipitation.

Representative mean concentration vs. time profiles (up to 6 h) of artemether an DHA of a pharmacokinetic interaction study between the antimalarial combination artemether/lumefantrine and combination antiretroviral therapy including nevirapine in HIV-infected adults, are presented in Fig. 6.

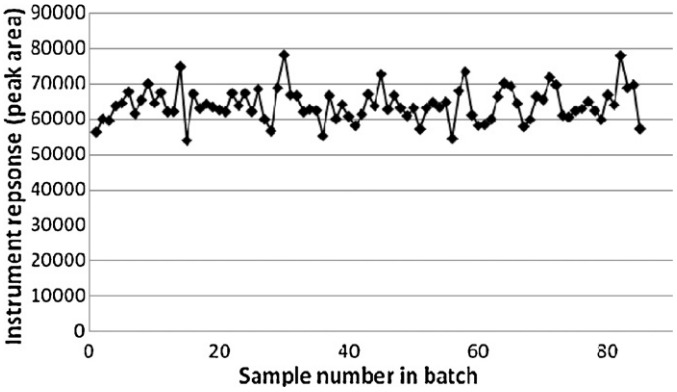

Fig. 4. Representative instrument response graph of the stable isotope labelled artemether internal standard for patient samples, standards and quality controls.

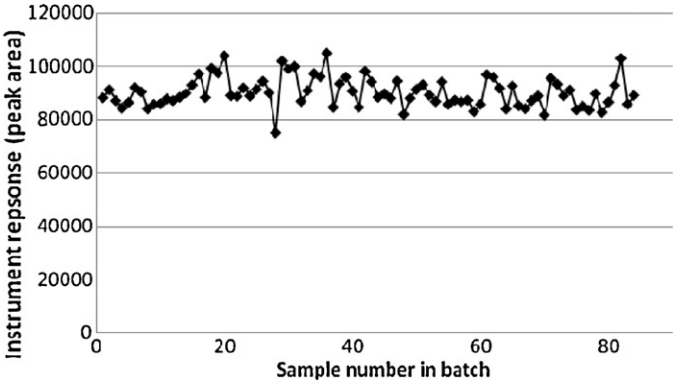

Fig. 5. Representative instrument response graph of the stable isotope labelled DHA internal standard for patient samples, standards and quality controls.

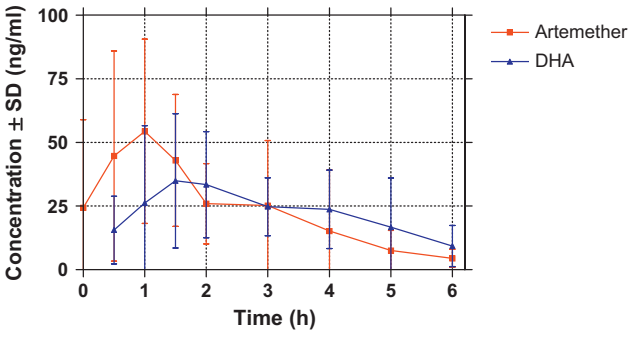

Fig. 6. Concentration vs. time profiles of artemether and DHA.

5. Conclusion

A robust solvent extraction assay was developed to measure artemether and DHA in malaria patient samples. The assay was used to quantify artemether and DHA in human plasma samples, which was generated from a safety and efficacy clinical trial in Mbarara, Uganda; as well as for a pharmacokinetic interaction study between the antimalarial combination artemether/lumefantrine and combination antiretroviral therapy including nevirapine in HIV-infected adults.

This assay withstands all the extraction and detection related pitfalls described by Lindegardh and co-authors for samples from patients with malaria, and although an organic solvent in a protein precipitation method may be problematic, exposure to an organic solvent in a liquid–liquid extraction (with isotope labelled internal standards) does not have the same effect. No significant suppression of ionisation was observed in the patient samples.

This assay method combined a simple and cost effective liquid–liquid extraction method with excellent PFP chromatography and MS/MS detection. Robust LC/MS/MS instrument performance was observed for standards, quality control standards, malaria free plasma samples, malaria infected plasma samples and haemolysed patient samples.

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References

[1] World Health Organization, World Malaria Report 2008, http://apps.who.int/malaria/wmr2008/malaria2008.pdf.
[2] H.K. Webster, E.K. Lehnert, Trans. R. Soc. Trop. Med. Hyg. 88 (1994) S27–29.
[3] W.H. Wernsdorfe, Expert Rev. Anti-infect. Ther. 2 (2004) 181–196, doi:10.1586/14787210.2.2.181.
[4] C. Souppart, N. Gauducheau, N. Sandrenan, F. Richard, Development and validation of a high-performance liquid chromatography–mass spectrometry assay for the determination of artemether and its metabolite dihydroartemisinin in human plasma, J. Chromatogr. B 774 (2002) 195–203.
[5] E. Peys, J. Vandenkerckhove, J. Van Hemel, B. Sas, Simultaneous determination of β-artemether and its metabolite dihydroartemisinin in human plasma and urine by a high-performance liquid chromatography–mass spectrometry assay using electrospray ionisation, Chromatographia 61 (2005) 637–641.
[6] B. Shi, Y. Yu, Z. Li, L. Zhang, Y. Zhong, S. Su, S. Liang, Quantitative analysis of artemether and its metabolite dihydroartemisinin in human plasma by LC with tandem mass spectrometry, Chromatographia 64 (2006) 523–530.
[7] I.R.S. Magalhãesa, V.A.P. Jabora, A.M. Fariab, C.H. Collins, I.C.S.F. Jardimc, P.S. Bonatoa, Determination of β-artemether and its main metabolite dihydroartemisinin in plasma employing liquid-phase microextraction prior to liquid chromatographic–tandem mass spectrometric analysis, Talanta 81 (2010) 941–947.
[8] J. Xing, H. Yan, R. Wang, L. Zhang, S Zhang, Liquid chromatography–tandem mass spectrometry assay for the quantitation of β-dihydroartemisinin in rat plasma, J. Chromatogr. B 852 (2007) 202–207.
[9] N. Lindegardh, W. Hanpithakpong, B. Kamanikom, P. Singhasivanon, D. Socheat, P. Yi, A.M. Dondorp, R. McGready, F. Nosten, N.J. White, N.P.J. Day, Major pitfalls in the measurement of artemisinin derivatives in plasma in clinical studies, J. Chromatogr. B 876 (2008) 54–60.
[10] L. Huang, A.L. Jayewardene, X. Li, F. Marzan, P.S. Lizak, F.T. Aweeka, Development and validation of a high-performance liquid chromatography/tandem mass spectrometry method for the determination of artemether and its active metabolite dihydroartemisinin in human plasma, J. Pharm. Biomed. Anal. 50 (2009) 959–965.
[11] I.C. Césara, J.A. de Aquino Ribeiroa, L. de Souza Teixeirab, K.B. Belloriob, F.C. de Abreub, J.M. Moreirab, P.R. Chellinia, G.A. Pianettia, Liquid chromatography–tandem mass spectrometry for the simultaneous quantitation of artemether and lumefantrine in human plasma: application for a pharmacokinetic study, J. Pharm. Biomed. Anal. 54 (2011) 114–120.
[12] Guidance for Industry Bioanalytical Method Validation, Department of Health and Human Services, Food and Drug Administration, Rockville, USA, 2001.
[13] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS, Anal. Chem. 75 (2003) 3019–3030.