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Application of an LC–MS/MS method for reliable determination of amodiaquine, *N*-desethylamodiaquine, artesunate and dihydroartemisinin in human plasma for a bioequivalence study in healthy Indian subjects

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ABSTRACT

A sensitive and high throughput bioanalytical method has been developed for reliable determination of amodiaquine (AQ), *N*-desethylamodiaquine (DEAQ), artesunate (AS) and dihydroartemisinin (DHA) in human plasma by LC–MS/MS. The method employs a solid phase extraction procedure without an evaporation step and with optimum use of organic solvents to circumvent degradation of artemisinin derivatives. The analytes and their deuterated internal standards (ISs) were analyzed on Hypersil Gold (100 mm × 4.6 mm, 5 μm) column using acetonitrile and 2.0 mM ammonium formate (pH 2.50) in 80:20 (*v/v*) ratio as the mobile phase. A triple quadrupole mass spectrometer equipped with an electrospray ionization interface was used to detect and quantify the analytes. The method was established over the concentration range of 0.250–30.0 ng/mL, 1.50–180 ng/mL, 2.00–600 ng/mL and 5.00–1400 ng/mL for AQ, DEAQ, AS and DHA respectively using 250 μL human plasma. The intra-day and inter-day accuracy and precision (% CV) across quality controls varied from 93.3–105.0% and 1.7–8.3 respectively for all the analytes. The stability was assessed in whole blood as well as in plasma samples under different conditions. All four analytes were stable in whole blood up to 2 h on melting ice. The long term stability in plasma was ascertained up to 90 days. IS-normalized matrix factors ranged from 0.988–1.023 for all the analytes. The method was successfully applied to a bioequivalence study using 50 mg artesunate and 135 mg amodiaquine fixed dose formulation in 14 healthy subjects.

1. Introduction

Malaria is a parasite disease that has caused major health concern worldwide. The *Plasmodium* species *P. falciparam* is the principal cause of malaria and has resulted in majority of death globally [1]. Moreover, the drugs used to treat uncomplicated malaria, a mild form of the disease have become ineffective in different parts of the world due to rapid development of resistance towards traditional antimalarial drugs such as chloroquine, sulfadoxine-pyrimethamine, amodiaquine and mefloquine [2]. World Health Organization (WHO) recommends artemisinin-based combination therapy (ACTs) for treating uncomplicated malaria. Artemisinin and its derivatives such as artesunate (AS), artemether and dihydroartemisinin (DHA) are antimalarials drugs with unique mode of action. These drugs have shown to produce faster relief of clinical symptoms and rapid clearance of parasites from blood compared to other antimalarial drugs [2,3]. However, they are not suitable for use as monotherapy due to their very short plasma half lives, and as a result require a long term dose regimen. Therefore, artemisinin derivatives are given with a long-acting drug with complimentary mode of action [2]. The artemisinin component is responsible for the reduction of the parasite biomass, while the companion drug clears the remaining biomass and thus offers protection against development of resistance. One such potent combination is that of AS and amodiaquine (AQ) which has shown good efficacy and tolerability for the first-line treatment of uncom-

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plicated malaria in different clinical studies [4–8]. A randomized trial was also conducted in Indian subjects for the first time to assess the safety and efficacy of this fixed dose combination [9]. The results showed acceptable efficacy for the treatment of *falciparam* malaria and the study proved that AQ is a suitable companion for AS.

AS is a hemisuccinate derivative of DHA, which is itself obtained by the reduction of artemisinin, a sesquiterpene lactone endoperoxide. The mechanism of action of AS involves splitting of its endoperoxide bridge by heme within the infected erythrocyte, giving rise to singlet oxygen. The parasite proteins, mainly in membranous structures are thus alkylated and are destroyed. After oral administration AS is extensively metabolized to its active metabolite DHA by blood esterases and some liver cytochromes, mainly CYP2A6. AQ is a synthetic 4-aminoquinoline antimalarial drug used to treat acute illnesses by destroying intraerythrocytic forms. Its activity is characterized by a schizonticidal action on *P. falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. AQ is rapidly absorbed after oral administration and transformed to its active metabolite *N*-desethylamodiaquine (DEAQ) [10].

Several bioanalytical methods are described to determine AS and its active metabolite DHA in different biological fluids [11–23]. Two other methods report simultaneous determination of artemether and DHA in human plasma [24,25]. Duthaler et al. [26] described an LC–MS/MS method for the simultaneous quantification of AS, DHA, artemether and its glucuronide metabolite in sheep plasma. Similarly, AQ and DEAQ have been determined as single analyte in human plasma [27] and human liver microsomes [28] respectively. Other methods report their simultaneous determination by either HPLC [29–32] or LC–MS/MS [33]. Concurrent analysis of AS, AQ and their active metabolites has been a subject of few reports [34–37]. Navaratnam et al. [34] analysed AS/DHA and AQ/DEAQ using two different HPLC methods with electrochemical detection. AS and DHA were studied in the reductive mode while AQ and DEAQ in the oxidative mode. Similarly, Fortin et al. [35] developed two different extraction protocols and optimized different chromatographic conditions for the analysis of AS/DHA and AQ/DEAQ respectively. Lai et al. [36] validated a solid-phase extraction procedure with different elution conditions for the determination of AS, DHA, AQ and DEAQ in human plasma by HPLC-with electrochemical detection. The calibration curves were linear in the concentration range of 2.0–1600 ng/mL for all the analytes and the method was applied to a pharmacokinetic study in healthy subjects. A promising single LC-tandem mass spectrometry method has been described for the simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma which are used in first-line combination treatments for malaria [37].

It has been shown that analysis of artemisinin derivatives in plasma is a challenging task due to several reasons such as haemolysis induced degradation of AS to DHA, interference of plasma phospholipids, and other issues related to selection of internal standard, effect of anti-coagulating agents and associated detrimental effect of organic diluents during sample preparation as highlighted by Lindegardh et al. [18]. Thus, the aim of the present work was to develop a sensitive, rugged and high throughput method for reliable determination of AS, AQ and their active metabolites in clinical samples.

2. Experimental

2.1. Chemicals and materials

Artesunate (AS, 99.83%) and amodiaquine hydrochloride (AQ, 99.91%) were purchased from Ipca Laboratories Ltd. (Mumbai, India), dihydroartemisinin (DHA, 99.10%) was procured from Mangalam Drugs and Organics Ltd. (Mumbai, India) and *N*-desethylamodiaquine dihydrochloride (DEAQ, 99.60%) was obtained from Neucon Pharma Pvt., Ltd. (Verna, Goa, India). Artesunate-d4 (AS-d4, IS, 100.00%) and dihydroartemisinin-13C, d4 (DHA-13C, d4, IS, 98.03%) were obtained from Vivan Life Sciences Pvt., Ltd. (Mumbai, India) while amodiaquine-d10 (AQ-d10, IS, 98.40%) and *N*-desethylamodiaquine-d5 dihydrochloride (DEAQ-d5, IS, 99.90%) were procured from TLC PharmaChem. Inc. (Ontario, Canada), HPLC grade methanol (MeOH) and acetonitrile (ACN) was product of J.T. Baker Inc. (Phillipsburg, NJ, USA). AR grade ammonium formate (AF) and formic acid (FA) were purchased from Qualigens Fine Chemicals (Mumbai, India). LiChrosep DVB-HL (30 mg/1.0 mL) solid phase extraction cartridges were obtained from Merck Specialties Pvt., Ltd. (Mumbai, India). Water was purified using Milli-Q water purification system from Millipore (Bangalore, India). Blank human blood was collected with Na heparin as anticoagulant from healthy and drug free volunteers. Plasma was separated by centrifugation at 2061*g* at 10 °C, and stored at −70 °C.

2.2. Liquid chromatographic and mass spectrometry equipment and conditions

The HPLC system from Shimadzu (Kyoto, Japan) consisted of a binary LC-20AD prominence pump, an autosampler (SIL-HTc), a solvent degasser (DGU-20A_3 prominence) and a temperature-controlled compartment for column (CTO-10AS_{VP}). The chromatographic analysis of the analytes and ISs was carried out under reversed-phase conditions at 30 °C using Hypersil Gold (100 mm × 4.6 mm, 5 μm) analytical column from Thermo Scientific (Cheshire, UK). The mobile phase consisted of ACN and 2.0 mM AF in water (pH 2.50, adjusted with FA) in 80:20 (*v/v*) ratio. For isocratic elution, the flow rate of the mobile phase was set at 0.6 mL/min. The autosampler temperature was maintained at 10 °C and the injection volume was kept at 5 μL. The pressure of the system was 1100 psi. The LC system was connected to a triple quadrupole mass spectrometer MDS SCIEX API-4000 (Toronto, Canada), equipped with electro spray ionization and operated in positive ionization mode. Optimized mass parameters and MRM transitions for analytes and ISs are given in Supplementary Table S1. Analyst classic software version 1.4.2 was used to control all parameters of LC and MS.

2.3. Calibrators and quality control samples

The plasma calibration standards (CSs) were prepared by spiking blank plasma with working solutions to obtain final concentration of 0.250, 0.500, 1.50, 3.00, 6.00, 12.0, 18.0, 24.0 and 30.0 ng/mL for AQ; 1.50, 3.00, 9.00, 18.0, 36.0, 72.0, 108, 144 and 180 ng/mL for DEAQ; 2.00, 4.00, 12.0, 60.0, 120, 240, 360, 480 and 600 ng/mL for AS and 5.00, 10.0, 30.0, 140, 280, 560, 840, 1120 and 1400 ng/mL for DHA respectively. Quality control samples were prepared at five concentration levels, lower limit of quantification quality control (LLOQ QC): 0.250/1.50/2.00/5.00 ng/mL; low quality control (LQC): 0.750/4.50/6.00/15.0 ng/mL; medium quality control-1 (MQC-1): 4.50/27.0/90.0/210 ng/mL; medium quality control-2 (MQC-2): 10.5/63.0/210/490 ng/mL; high quality control (HQC): 22.5/135/450/1050 ng/mL for AQ/DEAQ/AS/DHA respectively. All CSs and QC samples were prepared on ice with ice cold solutions to prevent any degradation of analytes as reported earlier [18]. The details of solution preparation of analytes and ISs are given in Appendix A—Supplementary data.

2.4. Extraction procedure

Prior to extraction, all frozen subject samples, CSs and QC samples were thawed in ice water bath and allowed to equilibrate at