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A high-performance liquid chromatography–tandem mass spectrometry method for the determination of artemether and dihydroartemisinin in human plasma

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ABSTRACT

A liquid chromatography–tandem mass spectrometry method is described for the quantitative determination of artemether (ART) and its metabolite dihydroartimisinin (DHA) in human plasma samples. Quantitation of ART and DHA in plasma is challenging due to the presence of malaria related hemolytic products in patient plasma causing degradation of the compounds when organic solvents are used during sample processing. Furthermore, both compounds consist of two epimeric forms that can interconvert both in solution and during chromatographic separation, an effect that is dependent on temperature and solvent properties and needs to be taken into account. This method utilizes micro-elution solid-phase extraction as sample preparation technique to minimize the need for organic solvents. Reversed-phase HPLC using a C18 50 × 2.1 mm column with 3.5 μm particles and a mobile phase of acetonitrile:water (30:70, v/v), followed by a step gradient at 90% acetonitrile, is applied to separate ART from DHA and matrix interferences within a run time of 4 min. Chromatographic conditions were optimized to allow analyte quantitation independent of the (unknown) ratio of the epimers in the injected sample. A triple quadruple mass spectrometer equipped with an atmospheric pressure chemical ionization interface in positive mode was used for detection in order to detect all epimeric forms. The method proved to be linear over a concentration range of 1.00–1000 ng/mL using 50 μL of plasma. Accuracy and precision were within 15% for bias and CV (20% at the lower limit of quantification). ART and DHA were stable (bias <15%) in plasma for 211 days after storage at −20 °C and −70 °C, 17 h on melting ice and 2 h at room temperature. Furthermore, both compounds were stable in whole blood after storage for 2 h on melting ice and at room temperature and after five freeze/thaw cycles. The method was successfully used for the analysis of pharmacokinetic samples originating from a drug–drug interaction study in which the antimalarial drugs artemether/lumefantrine were coadministrated etravirine or darunavir/ritonavir in healthy human immunodeficiency virus (HIV)-negative subjects.

1. Introduction

Artemether (ART, Fig. 1A) is a semi-synthetic derivative of artemisinin, a natural product, and widely used in malaria therapy. ART has an improved bioavailability compared to artemisinin and is therefore the most clinically used derivative. In vivo, it is rapidly metabolized to dihydroartimisinin (DHA, Fig. 1C) that also shows anti-malaria properties [1,2]. In order to support investigations of the pharmacokinetics (PK) of ART, selective, sensitive and reliable methods are needed to determine the concentrations of both ART and its metabolite in human plasma samples.

Several analytical methods have been published for the quantitative determination of artemisinin derivatives and their metabolite DHA in biological fluids and tissues. High performance liquid chromatography using ultraviolet (UV) detection with and without post-column derivatization [3–5] was frequently used but the unfavorable lower limit of quantitation (LLOQ), typically

Abbreviations: ART, artemether; DHA, dihydroartimisinin; PK, pharmacokinetics; UV, ultraviolet; LLOQ, lower limit of quantitation; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; MRM, multiple reaction monitoring; SPE, solid-phase extraction; HIV, human immunodeficiency virus.

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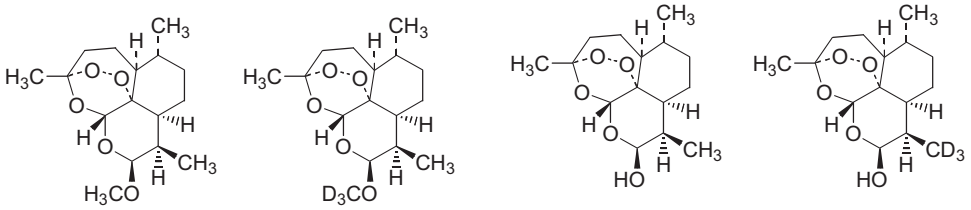

Fig. 1. Structures of artemether (A), artemether-d3 (B), dihydroartemisinin (C), and dihydroartemisinin-d3 (D).

10–50 ng/mL, and poor selectivity are inadequate for reliable quantification, especially at lower doses. The LLOQ can be reduced to 5 ng/mL by using electrochemical detection [6,7] but the methods are not straightforward as they require rigorously controlled anaerobic conditions and deoxygenation of the samples and mobile phases. A similar limit of detection was obtained using a GC method but required a plasma volume of 1 mL [8]. Over the last years, liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has been used for the determination of artemisinin derivatives and DHA with improved selectivity and sensitivity. LC–MS/MS with both electrospray ionization (ESI) [9–15] and atmospheric pressure chemical ionization (APCI) [16,17] has been applied successfully with quantitation limits of 1 ng/mL for ART and 2 ng/mL for DHA.

The quantification of ART and DHA is complicated by a number of special issues. In powder form, the steric configuration of the methoxy group of ART and the hydroxy group of DHA is in the β-position. In solution, however, the β-epimer of DHA is partially converted to the α-form, leading to a mixture of both lactol hemiacetal epimers at equilibrium [18]. This conversion from the β-position to the α-form is negligible for ART. The two epimers of DHA are separated using reversed-phase liquid chromatography [19] and also have different MS fragmentation properties [13] when ESI is used, and will, thus, behave as separate compounds. As a result, reliable LC–MS methods need to take this effect into account to ensure proper quantification of the amount of DHA. The rate and extent of the inter-conversion between the epimers is dependent on temperature, pH and polarity of the solvent [19]. Therefore, spiked plasma samples used as quality controls or calibrators may have a different ratio of the epimers compared to plasma obtained from patients or healthy volunteers during a clinical study. Moreover, previous studies have demonstrated that the inter-conversion can occur on a chromatographic timescale (i.e. on the HPLC-column) and, therefore, the ratio of the epimers in the injection solvent can be different from the ratio at the detector site [19]. Some published methods do not take this effect into account at all or consider the ratio constant and only use the α-DHA peak for quantification without further precautions [4,16,17]. The most applied bioanalytical strategy is to store processed samples overnight in the autosampler with the aim to reach equilibrium between the epimeric forms before injection. Quantification is then based on the α-DHA peak only and the β-DHA peak is disregarded [6,7,13–15,20]. However, this strategy only results in accurate results if the ratio of the epimers in the calibration, QC and patient samples is and remains the same during analysis. None of the published methods, however, have actually demonstrated this nor investigated the potential on-column epimerization which could result in inaccurate results if the rate is not similar for all samples. In a recent paper, Du et al. [10] used the sum of the peak areas of both epimers for quantification and thus addressed this issue, although the possible difference in response factor was not taken into account. Another complication is the observed large decrease of the ART and DHA response in hemolyzed plasma and plasma obtained from malaria patients. Ex vivo, unbound derivatives of artemisinin decompose in the presence of iron that is present in hemolyzed plasma [21]. Similarly, in the circulation of malaria patients, the malaria parasites consume hemoglobin and during this process release iron II-heme into the plasma compartment. Lindegardh et al. [22] demonstrated that this degradation only occurred during the sample pretreatment step when organic solvents were used such as during protein precipitation and was not related to instability in the matrix upon storage. They postulated that the organic solvent releases the analytes from their binding plasma proteins and the iron from the iron II-heme, enabling the iron to react with the analytes. Therefore, it is essential that methods for the determination of ART and DHA in malaria patient samples (or hemolytic plasma from healthy volunteers) are processed without the use of high amounts of organic solvents. Alternatively, the addition of sodium nitrite, a known methemoglobin-forming agent [23] or H_2O_2 [25] to plasma has been proposed to prevent degradation. This strategy was used in a few studies but its effect on the stability is not fully understood and instability still occurs.

The goal of our study was to develop a robust quantitative LC–MS/MS method capable of the simultaneous determination of ART and DHA in human plasma down to the 1 ng/mL level using 50 μL of plasma and taking all described complications into account. The influence of the mobile phase composition on the retention time and ratio of the epimeric forms was extensively investigated. The chromatographic and MS conditions were optimized to reliably quantify DHA, independent of the epimer ratio present in the samples. A solid-phase extraction sample treatment was developed, which allowed the extraction of ART and DHA from plasma with high recovery and prevented degradation of the compounds in hemolyzed plasma. Results of a thorough method validation in compliance with current FDA guidelines [24] are presented as well as the application of the validated method to the determination of ARM and DHA in plasma samples from a clinical study in which human volunteers were given a low dose (20 mg), resulting in ART and DHA concentrations in the low ng/mL range.

2. Experimental

2.1. Chemicals and reagents

Artemether was purchased from Sequoia Research Products (Pangbourne, United Kingdom) and dihydroartemisin, dihydroartemisin-d3 (Fig. 1B) and artemether-d3 (Fig. 1D) were purchased from Toronto Research Chemicals (Toronto, Canada). Methanol, (HPLC grade), acetonitrile (HPLC grade), ammonia, disodium hydrogenphosphate dihydrate, and formic acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q water system (Millipore, Bedford, MA, USA). DMSO was purchased from Sigma (Zwijndrecht, The Netherlands). K_2-EDTA plasma and whole blood were obtained from the clinical division of PRA-Early Development Services