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Development and validation of a liquid chromatography and ion spray tandem mass spectrometry method for the quantification of artesunate, artemether and their major metabolites dihydroartemisinin and dihydroartemisinin-glucuronide in sheep plasma

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Recently, promising fasciocidal activities of artesunate and artemether were described in rats and sheep. Therefore, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed to quantify artesunate, artemether and their metabolites dihydroartemisinin and dihydroartemisinin-glucuronide in sheep plasma. Protein precipitation with methanol was used for sample workup. Reversed-phase high-performance liquid chromatography (HPLC) was performed using an Atlantis C18 analytical column with a mobile phase gradient system of ammonium formate and acetonitrile. The analytes were detected by MS/MS using selected reaction monitoring (SRM) with electrospray ionisation in the positive mode (transition *m/z* 267.4 → 163.0). The analytical range for dihydroartemisinin, dihydroartemisinin-glucuronide and artesunate was 10–1000 ng/ml and for artemether 90–3000 ng/ml with a lower limit of quantification of 10 and 90 ng/ml, respectively. Inter- and intra-day accuracy and precision deviations were <10%. Consistent relative recoveries (60–80%) were observed over the investigated calibration range for all analytes. All analytes were stable in the autosampler for at least 30 h (6 °C) and after three freeze and thaw cycles. The validation results demonstrated that the LC–MS/MS method is precise, accurate and selective and can be used for the determination of the artemisinins in sheep plasma. The method was applied successfully to determine the pharmacokinetic parameters of artesunate and its metabolites in plasma of intramuscularly treated sheep.

Keywords: artemisinins; metabolites; glucuronide; fascioliasis; liquid chromatography–tandem mass spectrometry

Introduction

The endoperoxide artemisinin (qinghaosu), a secondary plant compound of the herb *Artemisia annua* (Chinese wormwood), is an efficacious and fast acting antimalarial drug.[1] Because artemisinin itself has biopharmaceutical shortcomings such as a poor bioavailability that limits its effectiveness, the sesquiterpene lactone scaffold of artemisinin was modified and the semi-synthetic derivatives have been developed. For example, the methyl ether derivative artemether (AM, Fig. 1) is characterised by a higher antiplasmodial activity than artemisinin.[1] The succinate derivative, artesunate (AS, Fig. 1), is water soluble and can be applied intravenously and is therefore indispensable for the treatment of cerebral malaria.[2] The semi-synthetic artemisinins are converted rapidly to dihydroartemisinin (DHA, Fig. 1) and are eliminated primary over bile by glucuronidation *in vivo* (Fig. 1).[1,3] Importantly, DHA and partly also the glucuronide adducts possess high-to-moderate antimalarial activity.[4]

The artemisinins do not only exhibit antiplasmodial but also trematocidal activities, since haemoglobin metabolism is common in *Plasmodia* and several trematodes including *Fasciola* spp.[5] The liver and bile parasitising food-borne trematodes *Fasciola hepatica* and *F. gigantica* are the causative agents of fascioliasis (fasciolosis). As many as 17 million people might be infected with *Fasciola* species. In addition, the veterinary impact is considerable as sheep, cattle and bovine are globally affected by the disease.[6,7] Currently, triclabendazole is the sole drug for the treatment of human fascioliasis. Additional drugs are commonly used in the treatment of veterinary infections with *Fasciola* spp. including albendazole, closantel, hexachlorophene, nitroxynil and rafoxanide. Resistance

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Journal of MASS SPECTROMETRY

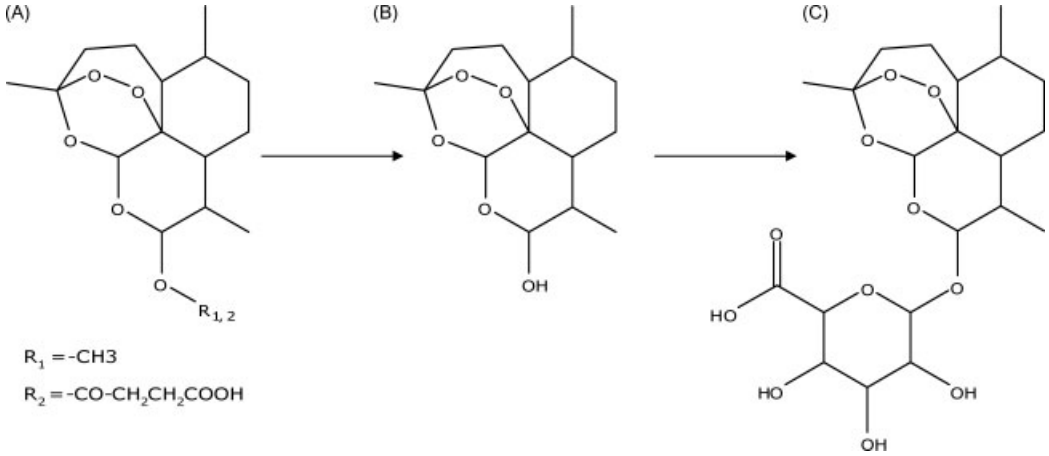

Figure 1. Hypothetical metabolic pathway of artemether and artesunate. (A) Parent drug: artemether (R_1, AM, molecular weight (MW): 298.37 g/mol) or artesunate (R_2, AS, MW: 384.42 g/mol). (B) Primary metabolite: dihydroartemisinin (DHA, MW: 284.35 g/mol). (C) Phase II metabolite: dihydroartemisinin-glucuronide (DHA-G, MW: 460.47 g/mol).

of triclabendazole and other trematocidals is widely spreading in livestock, but fortunately it has not been demonstrated in humans to date.[8] Promisingly, AM was effective against a triclabendazole-resistant *F. hepatica* strain in rats.[9]

Various analytical methods have been established in order to quantify the artemisinin derivatives in biological fluids and tissues. In the recent past, liquid chromatography–mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS/MS) methods were developed for experimental pharmacokinetic (PK) studies of α,β-arteether and DHA in rat plasma and serum.[10,11] In addition, different high performance liquid chromatography (HPLC) methods with mass spectrometric or electrochemical detection (HPLC-ECD) were developed for the measurement of AS or AM and DHA plasma levels in humans including a range of different sample procession methods (e.g. solid-phase extraction, liquid–liquid extraction and protein precipitation).[12–16] Lately, analytical methods were also developed for the simultaneous determination of up to 14 antimalarial drugs in human plasma since the artemisinins are nowadays routinely applied in combination with other antimalarial drugs to prevent resistance development.[17–20] However, to date no analytical method exists for the concurrent analysis of the semi-synthetic artemisinins, DHA and dihydroartemisinin-glucuronide (DHA-G).

Recently, we studied the efficacy and safety of single oral, intramuscular and intravenous dosages of AS and AM in *F. hepatica* infected sheep.[21,22] Plasma samples were collected at selected time points to support the drug efficacy study with PK data. We were interested to monitor the disposition of AS, AM as well as the two metabolites DHA and DHA-G, since experimental studies of humans and rats exposed to the artemisinins showed the presence of glucuronide metabolites in urine, bile and plasma.[23,24]

The aim of the present study was to develop and validate a LC–MS/MS method to quantify AS, AM and their metabolites DHA and DHA-G in sheep plasma for the prospective application to PK studies.

Experimental

Chemicals and reagents

AM and α,β-DHA were kindly provided from Dafra Pharma (Turnhout, Belgium). AS was obtained from Mepha AG (Aesch, Switzerland). Dihydroartemisinin-12-α-o-β-d-glucuronide (DHA-G) was purchased from Clearsynth (Mumbai, India). The chemical structures of AS, AM, DHA and DHA-G are depicted in Fig. 1. HPLC-grade acetonitrile and methanol were products of Bisolve (Valkenswaard, The Netherlands) and J.T. Baker (Deventer, The Netherlands), respectively. Ammonium formate (LC-MS grade), sodium nitrite, formic acid (98%, LC–MS grade) and acetic acid (glacial) were purchased from Fluka (Buchs, Switzerland). Potassium phosphate (KH_2PO_4), potassium hydroxide (reagent grade, >90%) and β-glucuronidase type IX-A from Escherichia coli (1 000 000–5 000 000 U/g protein) were obtained from Sigma–Aldrich (Buchs, Switzerland). Ultrapure water was produced with an Arium 61 215 water purification system (Sartorius Stedim Biotech, Göttingen, Germany) and applied for the preparation of mobile phase. Plasma from untreated sheep was collected in the framework of our PK studies in the Campania region of Italy.[21,22]

LC–MS/MS equipment and conditions

The high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) consisted of two LC-20AD pumps, a CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland), a thermostated Jones chromatography column oven (Omnilab, Mettmenstetten, Switzerland) and an online DG-3310 degasser (Sanwa Tsusho, Tokyo, Japan). Chromatographic separation was performed at 25 °C using a 2.1 mm × 20 mm Atlantis T3 3-µm analytical column (Waters, Milford, MA, USA). A 2.1 mm × 10 mm Atlantis 3-µm guard cartridge (Waters) was connected to the analytical column.

The mobile phase consisted of a mixture of 5 mM ammonium formate plus 0.15% (v/v) formic acid in ultra pure water (mobile A) and 0.15% (v/v) formic acid in acetonitrile (mobile B). The following stepwise gradient elution program was used: 0–1 min, B 2%; 1–10 min, B 2–45%; 10–13 min, B 45–50%; 13–15 min, B 50–95%; 15–20 min, B 95%; 20–21 min, B 95–2%; 21–22 min, B 2%. The gradient program allowed for a baseline separation of all analytes. Carryover (<0.1% carryover between subsequent analytical cycles) was managed by adding a wash cycle (95% mobile B for 5 min) at the end of each run. The flow rate of the mobile phases was set at 0.3 ml/min. Conditioning of the column was necessary to avoid shifts in retention time of certain analytes, i.e. AS, after a prolonged standby time of the instrument.

In more detail, the analytical column was conditioned with a replicate injection ($n = 6$) of the artemisinins standards (10 µg/ml in 1 : 1 methanol/mobile A) at the beginning of each series of experiments. The HPLC system was coupled to an API 365 triple-quadrupole mass spectrometer (PE Biosystems, Foster City, CA, USA) equipped with a turbo ion spray interface, operated in positive ionisation mode. A six-port switching valve (VICI Valco Instruments, Schenkon, Switzerland) was used to divert the effluent from the analytical column to the ion-spray interface of the mass spectrometer during 0–8 and 18–22 min of each run to avoid contamination of the mass spectrometer. The mass spectrometer was tuned by direct infusion (Harvard apparatus infusion pump 11, Massachusetts, USA) of 10 µg/ml of each artemisinin derivative solved in acetonitrile plus 0.1% (v/v) formic acid. All analytes were detected by selected reaction monitoring (SRM) with a transition of *m/z* 267.4 → 163.0. DHA-G, DHA and AS were analysed with a scan width and scan time of 3 amu and 2 s, respectively. AM was measured with a scan width and scan time of 5 amu and 3 sec, respectively. The major MS instrumentation settings are summarised in Table 1. Instrumentation control and data analyses were performed with Analyst 1.4.2. software (PE Biosystems) and Microsoft Excel 2003.

Standard, quality control and internal standard preparation

Standard stock solutions (1 mg/ml) were prepared in methanol. Appropriate volumes of stock solutions were serially diluted in a 1 : 1 mixture of methanol and mobile phase A to obtain working solutions of 0.3–30.0 µg/ml for DHA-G, DHA and AS and 2.8–90.0 µg/ml for AM. Plasma calibration samples were freshly prepared by diluting working solutions with blank sheep plasma (1 : 30, total volume of 300 µl), resulting in final concentrations of 1000.0, 400.0, 160.0, 64.0, 25.6 and 10.2 ng/ml for DHA-G, DHA and AS and 3000.0, 1500.0, 750.0, 375.0, 187.5 and 93.8 ng/ml for AM. Quality control (QC) samples were prepared with blank sheep plasma at low, medium and high concentrations (DHA-G, DHA, AS: 10.2, 160.0 and 1000.0 ng/ml; AM: 93.8, 750.0 and 3000.0 ng/ml). AS or alternatively AM was used as the internal standard (IS). Internal standard working solutions of 7.5 µg/ml AS or 150.0 µg/ml AM were diluted with blank sheep plasma (1 : 30, final concentration: 250.0 ng/ml or 5.0 µg/ml, respectively).

Table 1. LC–MS/MS instrumentation settings

Parameter	Value
Source temperature	400 °C
Nebuliser gas (NEB)	15 l/min
Curtain gas (CUR)	15 l/min
Declustering potential (DP)	26 V
Focusing potential (FP)	230 V
Entrance potential (EP)	6 V
Collision cell exit potential (CXP)	6 V
Ion spray voltage (IS)	5500 V
Collision gas N_2 (CAD)	3 l/min
Collision energy (CE)	13 eV
Polarity of analysis	Positive
Mass transition for the artemisinins	267.4 → 163.0 *m/z*

Plasma sample extraction procedure

An aliquot of 90 µl sodium nitrite solution (3 M) containing 1% (v/v) acetic acid was added to 300 µl of the sheep plasma samples and stirred (75 rpm) for 30 min at 37 °C in a TH/KS 15 incubator (Edmund Bühler, Hechingen, Germany). The addition of sodium nitrite was shown to prevent the degradation of the artemisinins in the presence of haemoglobin (Fe^{2+}-haeme) in haemolysed plasma samples.[19,25] The plasma–sodium nitrite mixture was vortex-mixed (Vortex Genius 3, IKA, Staufen, Germany) with 10 µl of IS working solution. For protein precipitation, 1000 µl of ice-cooled methanol was added to each sample and mixed for 1 min. The precipitated samples were cooled on ice for 10 min and subsequently centrifuged for 15 min at 16 100*g* (Eppendorf centrifuge 5415 R, Hamburg, Germany) at 4 °C. The supernatant was transferred to a 2-ml microtube and stored on ice. The pellet was vortex mixed with 500 µl of methanol for another minute and centrifuged as described above. The methanol extracts were combined, mixed and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 250 µl of methanol–mobile phase A (1 : 1, v/v), vortex mixed, centrifuged (15 min, 16 100*g*, 4 °C) and transferred to an autosampler vial. The autosampler rack was cooled at 6 °C. A 30-µl aliquot of each sample was injected into the LC–MS/MS system for analysis.

Calibration curves

Calibration curves were established using the internal standard method for the quantification of artemisinins plasma concentrations. Concentrations were plotted *versus* the peak-area ratios of the analytes to the IS. For α,β-DHA, the sum of the area under the curve of both anomers was used. Each calibration set consisted of one blank plasma sample (matrix sample processed without internal standard), one zero sample (matrix sample processed with internal standard) and six calibration samples including the lower limit of quantification (LLOQ). Concentrations chosen for the calibration curves ranged from 10.2 to 1000.0 ng/ml (DHA-G, DHA and AS) and 93.8–3000.0 ng/ml (AM). The six-point calibration standard curves were calculated and fitted either by linear (DHA-G and DHA) or quadratic regression (AS and AM). The Akaike information criterion (AIC) was used to select the most favourable type of regression for each calibration.[26] The quadratic regression model was applied because an increased signal strength was observed at higher concentration (fit by $y = ax^2 + bx + c$) and not because of saturation of the signal (i.e. fit by $y = -ax^2 + bx + c$). To determine the best weighting factor (none, $1/x$ or $1/x^2$), concentrations were back-calculated and the model with the lowest total bias across the concentration range was selected.

Glucuronide identification

A mass spectrometric approach followed by verification with an enzyme assay was conducted to assign and identify the glucuronide metabolite (DHA-G) in our sheep samples.

Plasma samples (10 × 300 µl) of AS or AM-treated sheep[21,22] were subjected to protein precipitation by the addition of 1000 µl ice-cooled methanol followed by centrifugation for 15 min at 16 100*g* at 4 °C. The supernatants were collected, stored at 4 °C and DHA-G was isolated by HPLC using the same chromatography program as described above (100 µl injection volume). Chromatography peaks representing DHA-G were collected on dry ice (retention time: 8.0–10.5 min). Collected samples were taken to dryness under nitrogen, resuspended in

Journal of MASS SPECTROMETRY

acetonitrile/formic acid 0.1% (v/v) and introduced into the MS/MS by infusion (10 µl/min). First, a Q1 single MS scan from *m/z* 100 to 1000 was carried out. MS conditions are described in Table 1 (CAD and CE were 0 l/min and 0 eV, respectively). Second, each signal with a relative intensity >30% was further examined using the artemisinin-specific transition *m/z* 267.4 → 163.0 in the SRM mode (CAD and CE were 3 l/min and 10–20 eV, respectively). Third, results were compared with reference spectra of DHA-G (10 µg/ml) obtained under identical conditions.

For the enzyme assay, the collected eluent was taken to dryness under nitrogen and resuspended in 1000 µl of 75 mM phosphate buffer, pH of 6.8, containing 1 mg/ml β-glucuronidase. This mixture was incubated for 120 min at 37 °C and agitated at 600 rpm using a Thermomixer (Eppendorf Thermomixer Comfort, Hamburg, Germany). The β-glucuronidase was inactivated by the addition of 1000 µl acetonitrile and the incubation mixture was analysed by LC–MS/MS. A 30-µl aliquot was measured before ($t = 0$) and after 120 min exposure to β-glucuronidase.

Method validation

Our method was validated for selectivity, precision, accuracy, recovery and stability according to the bioanalytical method validation guidance for industry 2001 by the Food and Drug Administration (FDA).[27]

Selectivity

Blank plasma obtained from sheep of different origin ($n = 6$) was examined for interferences with endogenous substances using the above-described extraction procedure but without adding the IS working solution.

Lower limit of quantification

The LLOQ was selected as the minimal concentration in plasma samples, which could be analysed with a precision of ≤20% and accuracy between 80% and 120%. In addition, the analyte response was at least five times above the noise level of the blank response.

Accuracy and precision

Accuracy and precision of the method was evaluated by analysing QC samples ($n = 5$) at the LLOQ, a medium concentration (middle QC) and at the upper limit of quantification (ULOQ). The intra- and inter-day accuracy/precision was determined within a single run and between different assays ($n = 3$), respectively. The following concentrations were selected for QC samples, covering the entire range of the calibration curve: 93.8, 750.0 and 3000.0 ng/ml for AM and 10.2, 160.0 and 1000.0 ng/ml for DHA-G, DHA and AS. Freshly prepared calibration standards were used for analyses. The precision was calculated using the coefficient of variation (CV [%]). The accuracy was determined as the percentage ratio of the measured concentration to the nominal concentration. A precision of ±15% (LLOQ: ±20%) and accuracy between 85% and 115% (LLOQ: 80–120%) was accepted in our study.

Recovery and matrix effect

Relative recoveries (RRE) of the artemisinin derivatives were determined by comparing the absolute peak areas of blank plasma samples spiked before and after the extraction at three different concentrations ($n = 3$ per concentration). The recovery of AM was investigated at 93.8, 750.0 and 3000.0 ng/ml and the recovery of DHA-G, DHA and AS at 10.2, 160.0 and 1000.0 ng/ml.

Matrix effects of the artemisinins were assessed as the ratio of the absolute peak areas of blank plasma samples spiked after the extraction to the absolute peak areas of the analytes solved in a mixture of methanol–mobile A (1 : 1, v/v).

Stability: autosampler and freeze–thaw stability

Autosampler stability and freeze–thaw stability studies were included in our method validation. QC samples at low, medium and high concentration (as described above) were used to test stabilities under different conditions. Autosampler stability was evaluated by the analysis of QC samples ($n = 3$ per concentration) over a period of 30 h. For the freeze–thaw stability, QC samples were frozen at −80 °C (1 h) and thawed at room temperature (1 h). This cycle was repeated three times before analysis. The concentrations of these samples were compared with concentrations of freshly prepared QC samples. The drug solutions were considered as stable with a deviation of not more than ±15% and ±20% at the LLOQ.

Sample dilution

PK samples with drug concentrations exceeding the ULOQ were diluted using blank sheep plasma. The dilution effect was evaluated as described by Hodel *et al.*[19] to assure that the accuracy of the analysis was not affected. In brief, blank sheep plasma ($n = 3$) was spiked with a concentration exceeding two times the ULOQ. The samples were then fourfold diluted with blank plasma to achieve a concentration within the calibration range. The accuracy of drug quantification was defined as the percentage ratio of the measured concentration to the nominal concentration. A deviation of ±15% of the measured concentration from the nominal value was accepted in our study.

Pharmacokinetic study

The PK studies were carried out in the framework of two efficacy studies published recently.[21,22] Here, we present the PK profile of one sheep treated with 60 mg/kg AS intramuscularly to demonstrate the usefulness of the developed and validated LC–MS/MS method. Blood samples were withdrawn from the jugular vein into lithium-heparin-coated vacutainer tubes (BD, Franklin Lakes, NJ, USA) at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 6, 8 and 24 h posttreatment. Plasma was produced by centrifugation and stored at −80 °C. AM (5.0 µg/ml) was used as IS for the analysis. PK samples with drug concentrations exceeding the ULOQ were diluted with blank sheep plasma as described above.

Results and Discussion

Method development

To date, no validated analytical method exists for the simultaneous quantification of the antimalarials AS, AM and their metabolites DHA and DHA-G for the analysis of plasma samples from PK studies. We, therefore, decided to develop a LC–MS/MS method for the quantification of these drugs and metabolites in plasma samples obtained from *F. hepatica*-infected sheep. Importantly, DHA-G seems to be the major glucuronide metabolite of DHA and the primary elimination occurs over the bile.[3] Furthermore, since adult

F. hepatica live in the bile ducts, biliary elimination of potentially active drug metabolites might even enhance drug activity.

In a first step, MS/MS conditions were defined and optimised. Stock solutions in acetonitrile of each analyte were directly infused and single MS scans were carried out in the positive ion detection mode. Base peaks with good responses were observed at *m/z* 221.5, 267.4 and 284.4, corresponding to protonated fragments of the parent molecules of AS, AM and DHA. In order to increase the sensitivity and selectivity of the MS signal, the common fragment with the best response (*m/z* 267.4) was selected and subjected to further fragmentation. Best results for all analytes of interest were obtained using the transition *m/z* 267.4 → 163.0. The MS/MS parameters were adjusted to maximise the amount of protonated fragment ion (163 *m/z*) to achieve the highest sensitivity (Table 1).

Next, the LC conditions were refined. Different analytical columns, which had been used previously for the separation of the artemisinins and synthetic peroxides,[15,19,28] were tested. A major challenge was the baseline separation of DHA and AS. This could be achieved using all tested columns, i.e. the X Terra (C18, 2.1 mm × 150 mm, 5 µm), the Phenomenex (C8 (2), 2.0 mm × 50 mm, 5 µm) and the Atlantis (C18, 2.1 × 20, 50 and 100 mm, 3 µm). However, an additional separation of the two DHA anomers (α,β-DHA) was best achieved using the Atlantis column (C18, 2.1 mm × 20 mm, 3 µm). In addition, good peak symmetry of α,β-DHA was obtained with this column (Fig. 2(F)). An increase in the column length from 20 to 100 mm did not offer additional advantages. A slow gradient of mobile B (acetonitrile plus 0.15% (v/v) formic acid) from 2 to 95% over 15 min was required for the separation of β-DHA and AS. A wash cycle of 5 min at 95% mobile B and an additional equilibration step to 2% mobile B was necessary to avoid carryover effects (<0.1%). It might be possible to shorten the run time of 23 min using chromatography columns with smaller particles (e.g. pellicular silica columns) or column switching,[29,30] in case a need of high-throughput analysis would arise. Variations in the ammonium formate concentration (0, 5, 10, 20 and 50 mM) of mobile A did not affect the separation; hence, 5 mM ammonium formate plus 0.15% formic acid was used, which

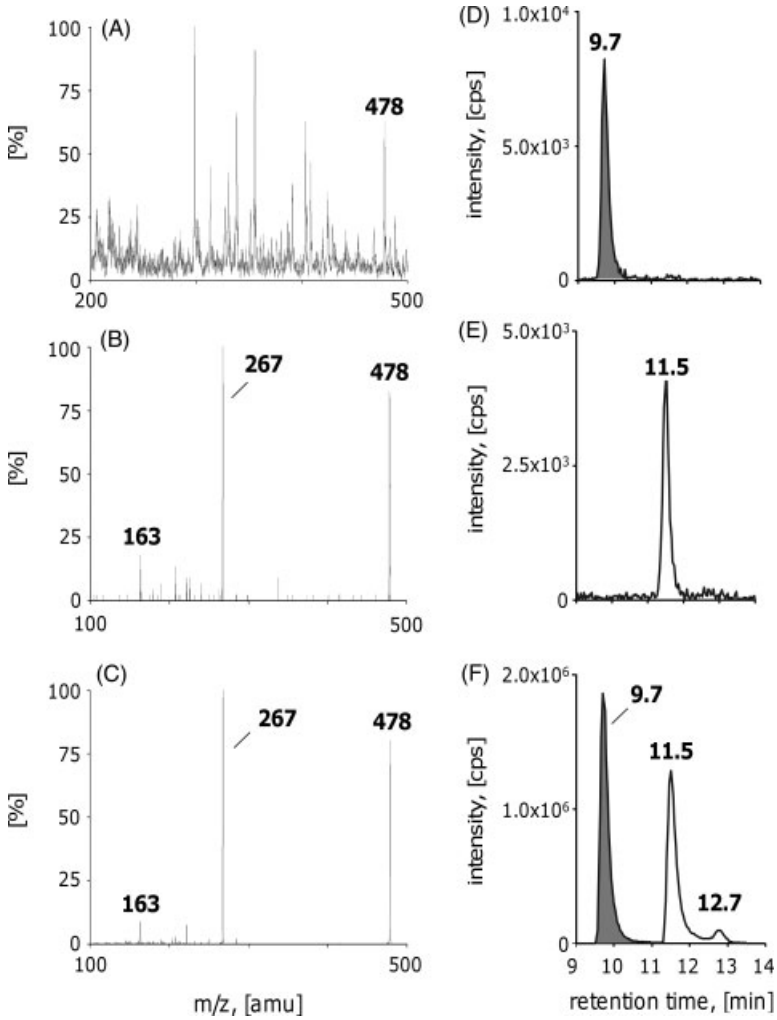

Figure 2. Chromatographic separation of DHA-G and α,β-DHA and confirmation of the identity of DHA-G in sheep plasma. (A) Single MS (Q1 positive mode) spectrum of the collected DHA-G chromatographic peak. The ammonium ion adduct of DHA-G (DHA-G-NH_4^+) is characterised by *m/z* 478. (B) Tandem MS (MS/MS positive mode) selected reaction monitoring of parent *m/z* 478. Characteristic artemisinins-scaffold fragments *m/z* 267 and 163 are shown. (C) Selected reaction monitoring of DHA-G (*m/z* 478) reference compound leads to an identical fragmentation pattern as shown in (B). (D) Chromatogram of isolated metabolite DHA-G (retention time: 9.7 min; grey peak). (E) Chromatogram of isolated metabolite DHA-G after incubation with β-glucuronidase giving rise to DHA (retention time: 11.5 min; white peak). (F) Chromatogram of reference compounds. DHA-G: retention time = 9.7 min, grey peak. α-DHA: retention time = 11.5 min, white peak. β-DHA: retention time = 12.7 min, white peak.

Journal of MASS SPECTROMETRY

achieved the best peak symmetry and a good ratio of height to width.

Finally, different sample workup methods were tested for simplicity, robustness and recovery of analytes from plasma. A liquid–liquid extraction method with ethyl acetate or ether[15,31] was found to be laborious and recovery rates were not superior to a plasma protein precipitation with methanol or acetonitrile. A precipitation with methanol at a ratio of 3 : 1 (precipitant to plasma) provided the highest recovery rates of all analytes. An improved sensitivity and signal-to-noise ratio was achieved when the supernatant was evaporated under a stream of nitrogen and resuspended in a smaller volume of methanol and mobile phase A (50 : 50, v/v).[19]

Glucuronide identification

We identified an unknown metabolite in plasma samples of AS and AM-treated sheep, which was suspected to represent the phase II metabolite DHA-G (Fig. 1). The collected metabolite fraction contained neither DHA, AS nor AM as demonstrated using LC–MS/MS. The absence of DHA was a prerequisite to be able to carry out β-glucuronidase biotransformation experiments *in vitro*. The retention time of the metabolite was 9.7 min (Fig. 2(D)) and was characterised by *m/z* 478 (Fig. 2(A)), corresponding to the ammonium ion (*m/z* 18) adduct of DHA-O-glucuronide (DHA-G, *m/z* 460). In Q1 negative mode, the parent molecule (free acid of DHA-G) was apparent with *m/z* 459. Fragmentation of the isolated metabolite gave rise to two characteristic fragments with *m/z* 163.0 and 267.4 (Fig. 2(B)). These fragments were identical to the typical artemisinins scaffold fragments identified during method setup (see above). It is important to note that only mass *m/z* 478 (out of all signals with a relative intensity >30%) was characterised by the transition *m/z* 267 → 163. The identity of the collected metabolite DHA-G was further confirmed by enzymatic digestion using β-glucuronidase. By this procedure, DHA-G was converted completely to DHA (Fig. 2(D and E)). This conversion was observed in the presence of β-glucuronidase only, but not in control incubations, which were carried out in absence of the enzyme. Finally, DHA-G reference compound was obtained and used as an additional control. The fragmentation pattern of this compound was identical to the fragmentation pattern of the isolated metabolite (Fig. 2(C)). Furthermore, retention times of DHA-G and DHA (Fig. 2(F)) matched the retention times of the isolated metabolite or DHA-G reference compound prior (Fig. 2(D)) and after (Fig. 2(E)) enzymatic digestion. We conclude that DHA-G is a major metabolite of AS and AM in sheep.

Method validation

Selectivity and LLOQ

Blank sheep plasma samples ($n = 6$) of different origin were analysed for the presence of interfering endogenous matrix components. No endogenous peaks were detected in any of the six blank plasma samples at the retention time of DHA-G (9.7 ± 0.5 min), DHA (11.5 ± 0.5 min), AS (13.7 ± 0.5 min) or AM (16.0 ± 0.5 min) as demonstrated in Fig. 3(A). We conclude that the present method is selective.

The LLOQ for DHA-G, DHA and AS was set at 10.2 ng/ml. The obtained LLOQ for AM was 93.8 ng/ml, which was considered to be acceptable. The signal-to-noise ratio at LLOQ was 5 : 1. Representative chromatograms of processed blank sheep plasma spiked with the four artemisinins at LLOQ are depicted in Fig. 3(B and C).

Calibration curve

The AIC was used to select the most favourable type of regression for each analyte.[26] The calibration curves of DHA-G and DHA were best fitted with a linear model over a concentration range from 10.2 to 1000.0 ng/ml in sheep plasma. The coefficients of correlation (r^2) were >0.998. The calibration standards of AS and AM were analysed using a quadratic regression model over a range of 10.2–1000.0 and 93.8–3000.0 ng/ml, respectively ($r^2 > 0.998$). According to the regressions used, the accuracy of the back-calculated concentration standards was better than 100 ± 10% of the nominal concentration in each experiment. This value is within the recommended limit of 100 ± 15%.[27]

Accuracy and precision

Inter- and intra-day precision and accuracy were calculated by analysing five QC samples at three concentrations on three different days. For the intra-day precision and accuracy, a representative experiment is shown ($n = 5$ measurements, Table 2). For the inter-day precision and accuracy, three independent sets of experiments are summarised in Table 2 ($n = 15$ measurements). The mean relative standard deviation (RSD) values in the intra- and inter-day

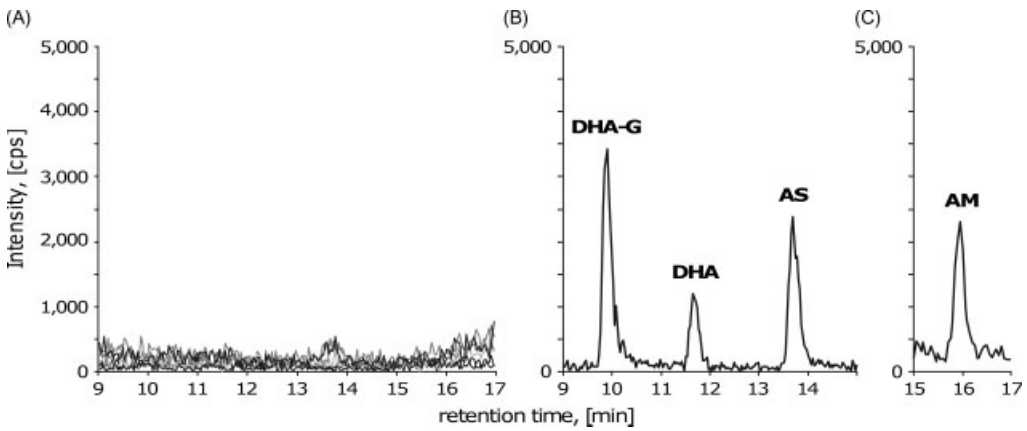

Figure 3. Chromatograms illustrate the selectivity and the analytes LLOQ of the analytical method. (A) Chromatograms of extracted blank sheep plasma samples ($n = 6$). (B) Chromatogram of the LLOQ of DHA-G, DHA and AS (10.2 ng/ml). (C) Chromatogram of the LLOQ of AM (93.8 ng/ml).

Table 2. Intra-day and inter-day accuracy and precision

Analytes (regression model)	Nominal concentration (ng/ml)	Intra-day[a] Mean concentration found (ng/ml)	Intra-day[a] RSD (%)	Intra-day[a] Accuracy (%)	Inter-day[b] Mean concentration found (ng/ml)	Inter-day[b] RSD (%)	Inter-day[b] Accuracy (%)
Artemether (quadratic, $R^2 = 0.9998$)	93.8	93.0	5.2	99.1	94.8	9.2	101.1
	750.0	751.0	3.0	100.1	735.4	7.5	98.0
	3000.0	3075.7	3.3	102.5	2898.7	7.4	96.6
Artesunate (quadratic, $R^2 = 0.9999$)	10.2	10.5	4.0	103.4	10.6	6.0	103.6
	160.0	155.2	3.2	97.0	163.6	6.3	102.3
	1000.0	942.1	2.6	94.2	971.8	3.8	97.2
Dihydroartemisinin (linear, $R^2 = 0.9993$)	10.2	10.8	3.2	106.1	10.6	5.3	104.2
	160.0	159.9	4.7	99.9	161.4	4.3	100.9
	1000.0	1011.0	3.8	101.1	998.7	4.7	99.9
DHA-glucuronide (linear, $R^2 = 0.9991$)	10.2	10.3	8.6	100.5	10.3	7.7	100.6
	160.0	161.2	2.6	100.7	165.7	6.0	103.6
	1000.0	993.8	3.4	99.4	997.1	3.4	99.7

[a] Values are means of $n = 5$ samples. A representative experiment is shown.
[b] Values are means of $n = 3$ independent sets of experiments.

precision runs of all analytes were 4.0% (range: 2.6–8.6%) and 6.0% (range: 3.4–9.2%), respectively. The intra- and inter-day accuracies ranged from 94.2% to 106.1% and 96.6% to 104.2%, respectively. These data indicate that the developed method is very reliable.

Extraction recovery and matrix effect

RRE of the artemisinins were determined by comparing the absolute signal of blank plasma samples spiked before and after extraction at low, medium and high concentrations ($n = 3$). As shown in Table 3, mean recoveries of 83.9%, 77.7% and 78.9% were observed for DHA-G, DHA and AS, respectively. The mean recovery of AM (57.8%) was about 20% lower compared with the other analytes. The CV of the recoveries over the entire concentration range was <6.7%, indicating consistency in our sample processing method.

Matrix effects were assessed as the ratio of the absolute peak areas of blank plasma samples spiked after extraction to the absolute peak areas of the analytes solved in a mixture of mobile A and methanol (1 : 1, v/v) (Table 3). The matrix effects were not significant for DHA-G (83.9%) and AS (93.3%). On the other hand, a considerable loss of signal was seen for DHA and AM in sheep plasma (matrix effect of 57.7% and 63.2%, respectively). The matrix effect of DHA was independent of the analyte concentration with a CV of 4.4% over the entire concentration range and of the plasma batches. In contrast, the matrix effect was highly dependent on analyte concentrations of AM (CV 19.62%), with a particularly high suppression observed at low AM concentrations but was not affected by the plasma source. Different operational strategies have been suggested such as extensive cleanup procedures (solid phase or liquid–liquid extraction) or more efficient chromatographic separations to minimise the interferences of coeluting matrix compounds.[32] However, our method achieved consistent results over the course of several experiments. Since the sensitivity of our method was considered to be sufficiently high, no additional sample workup procedures were implemented.

Table 3. Relative recovery (RRE) and matrix effect of artesunate, artemether and its metabolites dihydroartemisinin and DHA-glucuronide

Analytes	Nominal concentration (ng/ml)	RRE (%)[a]	Mean ± CV (%)	Matrix effect (%)[a]
Artemether	93.8	61.8	57.8 ± 6.7	51.5
	750.0	57.4		61.9
	3000.0	54.1		76.2
Artesunate	10.2	79.3	78.9 ± 0.5	111.9
	160.0	78.5		89.6
	1000.0	78.9		78.3
Dihydroartemisinin	10.2	74.6	77.7 ± 5.2	55.6
	160.0	76.3		60.3
	1000.0	82.4		57.2
DHA-Glucuronide	10.2	83.3	83.9 ± 2.7	91.8
	160.0	82.0		79.9
	1000.0	86.3		80.0

[a] Values are means of $n = 3$ samples.

Stability

Results obtained from autosampler and freeze–thaw stability studies demonstrated that the samples were stable under these conditions (Table 4). The variations of all target drugs in processed plasma samples kept at 6 °C for 30 h were below 9.8%. The accuracies under these conditions ranged from 88.1% to 111.2%. The samples were highly stable following several complete freeze/thaw cycles (accuracies: 91.0–107.5%; RSD <13.4%). Previous studies have shown that AS and DHA are not stable at room temperature.[19] Similarly, in our experiments a substantial variability of DHA-G precision was observed if samples were kept at room temperature. These effects were particularly pronounced at low concentrations (data not shown). In light of these findings, samples were processed on ice and stored at low temperatures.

The succinate functional group of AS is vulnerable to chemical hydrolysis or enzymatic degradation by means of plasma

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Table 4. Stability analysis of quality control samples for artesunate, artemether, dihydroartemisinin and DHA-glucuronide in sheep plasma ($n = 3$)

Experiment	Analytes	Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	RSD (%)	Accuracy (%)
Autosampler	Artemether	93.8	88.5	3.4	94.4
		750.0	809.4	4.4	107.9
		3000.0	3021.8	7.2	100.7
Autosampler	Artesunate	10.2	11.3	2.4	111.2
		160.0	168.5	5.4	105.3
		1000.0	1007.7	3.0	100.8
Autosampler	Dihydroartemisinin	10.2	9.0	2.1	88.1
		160.0	166.8	1.4	104.3
		1000.0	1107.5	6.2	110.7
Autosampler	DHA-glucuronide	10.2	11.2	9.8	109.8
		160.0	159.6	4.0	99.7
		1000.0	998.3	4.8	99.8
F/T	Artemether	93.8	89.9	5.2	95.8
		750.0	682.6	7.4	91.0
		3000.0	2736.3	6.9	91.2
F/T	Artesunate	10.2	9.2	7.7	90.0
		160.0	167.7	13.4	104.8
		1000.0	1075.1	4.4	107.5
F/T	Dihydroartemisinin	10.2	10.1	0.3	99.2
		160.0	152.5	1.4	95.3
		1000.0	988.1	6.4	98.8
F/T	DHA-glucuronide	10.2	10.5	8.4	103.3
		160.0	158.4	2.5	99.0
		1000.0	975.8	6.4	97.6

Autosampler, samples were kept in the autosampler at 6 °C for 30 h after processing; F/T, stability after three freeze–thaw cycles.

esterases[33] (Fig. 1). We observed that on average 5.5% (95% CI: 5.2–5.8%) of AS reacted to DHA during sample workup. This degradation should be kept in mind, in particular, if in an analysed specimen the ratio between the AS signal and DHA signal is very high. Note that the endoperoxidic artemisinins degrade in the presence of Fe^{2+} in haemolysed plasma samples. The addition of sodium nitrite, a known methaemoglobin-forming agent, to all plasma samples successfully prevented this reaction in recent studies and had no impact on the results in non-haemolysed plasma samples.[19,25,34]

Sample dilution

Spiked plasma samples ($n = 3$) at a concentration twofold of the ULOQ were diluted fourfold with blank plasma. The mean deviations (bias) from the nominal concentrations were 7.7%, 5.4%, 7.5% and 7.2% for AS, AM, DHA and DHA-G, respectively. In conclusion, plasma samples containing high artemisinin concentrations (above the ULOQ) can be diluted with blank plasma without affecting the accuracy of the measurement.

Method application

The validated method was applied to analyse plasma samples from one *F. hepatica*-infected sheep treated intramuscularly with 60 mg/kg of AS.[22] AM (5 µg/ml) was used as the internal standard. DHA-G eluted at 9.9 min, followed by DHA at 11.7 min, AS at 13.8 and AM at 16.0 min, which is in line with spiked reference plasma samples (Fig. 4(A and B)). The concentration *versus* time profile of one AS-treated sheep is depicted in Fig. 4(C). In this sheep AS reached a maximal plasma concentration (C_{max}) of 6669 ng/ml 15 min posttreatment, followed by DHA (C_{max} = 1525 ng/ml) and DHA-G (C_{max} = 18 526 ng/ml) at 30 and 45 min posttreatment, respectively. This result reflects the suggested metabolism pathway of AS (Fig. 1): AS is converted to DHA by ester cleavage and subsequently metabolised by *o*-glucuronidation to DHA-G. Glucuronidated DHA was found to be a prominent main metabolite, which showed a three times higher C_{max} than the parent compound AS. Based on these results, the trematocidal activity of the main metabolite DHA-G will be explored in more detail.

Conclusion

A LC–MS/MS method was developed and validated for the simultaneous quantification of AS or AM and their metabolites DHA and DHA-G. The method has proven to be selective, precise, accurate and simple to perform. The sensitivity for all analytes was in the range of 10–90 ng/ml. The method was applied successfully to analyse plasma samples of AS-treated sheep and will be used in the future for detailed PK studies. For the first time, *o*-glucuronidated DHA (DHA-G) was described to be a main phase II metabolite of AS in sheep. This method will be adapted to analyse additional biofluids (e.g. bile) or tissues (e.g. liver) in different species including humans to explore pharmacological effects of artemisinins in *F. hepatica*-infected individuals.

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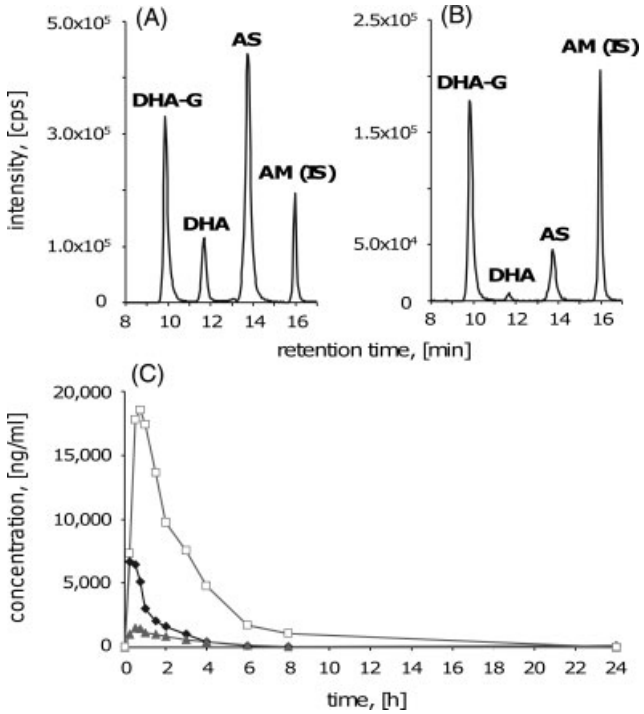

Figure 4. Plasma-concentration profile of an AS-treated sheep infected with *F. hepatica*, illustrated as an application of the method. (A) Reference chromatogram of DHA-G, DHA and AS 1000.0 ng/ml and AM (IS) 5.0 µg/ml spiked blank sheep plasma. (B) Chromatogram of a plasma sample 60 min after intramuscular administration of AS 60 mg/kg (diluted with blank plasma 1 : 30). (C) Plasma concentration–time profile of AS (□), DHA (♦) and DHA-G (▲) in sheep following intramuscular administration of 60 mg/kg AS.

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