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Direct analysis of artemisinin in plasma and saliva using coupled-column high-performance liquid chromatography with a restricted-access material pre-column

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Abstract

A previously established HPLC system with post-column derivatization for the analysis of artemisinin was coupled to an ADS (alkyl-diol silica) pre-column, allowing direct and repetitive injection of protein-rich fluids such as plasma. The limit of quantitation for 100 μl of plasma was 10 ng/ml (CV=10.5%) while concentrations down to 2 ng/ml could be quantified for 1.00 ml saliva samples (CV=11.1%). The system was linear in the tested range of 10–2000 ng/ml for plasma and 2–240 ng/ml for saliva samples, respectively. This paper introduces coupled column HPLC as a simplified method for the routine analysis of artemisinin in biological fluids.

Keywords: Artemisinin

1. Introduction

Artemisinin (Fig. 1), extracted from the Chinese medical plant qinghao (*Artemisia annua* L.), represents a class of antimalarials that have successfully been used for more than two decades for treatment of malaria in regions with multi-drug resistant *Plasmodium falciparum* [1]. However, documentation of these endoperoxide antimalarials has been delayed by the lack of concerted development. Until the mid 1990s, clinical pharmacokinetic studies of artemisinin and its semi-derivatives (dihydroartemisinin, artesunic acid, artemether, arteether) were scarce, largely due to the lack of reliable and sensitive methods for their quantitation in biological fluids. The presently emerging pharmacokinetic information has mainly been based on methods employing either potentiometric detection, first described by Zhou [2] and later validated in a number of laboratories for various derivatives [3–10], or post-column on-line derivatisation with UV detection [11] with subsequent modifications [12–14]. In terms of sensitivity, the two methods are comparable for the quantitation of artemisinin, whereas lower units of quantitation for most semi-synthetic derivatives are reached with electrochemical detection [15]. On the other hand, the high potential in the reductive mode, resulting in noise and baseline drift due to the

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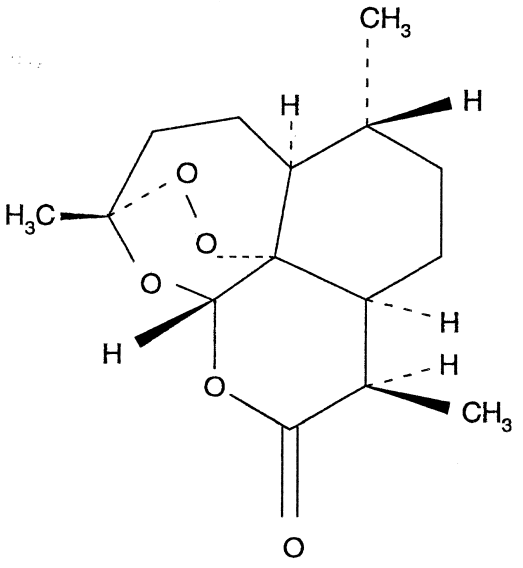

Fig. 1. Structural formula of artemisinin (molecular weight 282).

presence of oxygen in the chromatographic system, can be a limiting factor in the routine analyses of large sample sizes such as those seen in pharmacokinetic studies. Although less suitable for quantitation of ether derivatives such as artemether and arteether, on-line post-column alkali derivatisation with UV detection for artemisinin is a robust method and, importantly, amenable to automation. Other methods [16–21] have yet to be applied to clinical studies.

Average maximum plasma concentrations after single oral clinical doses of 500 mg range between 400 and 700 ng/ml [22,23]. Lower maximum concentrations (100–185 ng/ml) are seen after rectal administration [24,25]. The biological half-life in patients is about 2 h. Artemisinin concentrations in saliva are in good agreement with unbound plasma levels [26].

For routine quantitation of drugs in biological fluids by liquid chromatography (LC), batch extraction prior to column separation is a time- and resource-consuming step. Coupled column LC systems offer the potential advantage of direct quantification of samples of biological fluids without off-line sample clean-up. In such a system, the target molecules are first retained on a sample-processing precolumn before separation on the analytical column. Special chromatographic supports have been developed as packing material for these pre-columns [27,28]. These restricted-access material (RAM) columns exclude macromolecules such as plasma proteins without causing their denaturation whilst small hydrophobic molecules are retained. A commercially available column with such a packing material is the alkyl-diol silica (ADS) column.

We have developed an on-line sample preparation system for the quantitation of artemisinin in human saliva and plasma, thereby reducing the time and labour necessary for the analysis of the numerous samples derived from pharmacokinetic investigations. The presented method is based on a post-column derivatization system that was previously reported [11] with several modifications including the introduction of the on-line RAM column, changes in the flow of mobile phases and concentration of the post-column alkali solution.

2. Experimental

2.1. Reagents and materials

Artemisinin for preparation of stock solutions was obtained from the Institute of Materia Medica (Hanoi, Vietnam). NMR (400 MHz) analysis showed a 99.5% purity for the compound. Ethanol was purchased from Kemetyl (Stockholm, Sweden). All other chemicals were of analytical grade and were purchased from Merck (Germany) if not stated otherwise. All water for the system was purified by a Milli-Q Academic gradient (Millipore, Bedford, MA, USA).

2.2. Chromatographic instrumentation

The system consisted of three HPLC pumps, a LC-10AD from Shimadzu (Shimadzu, Kyoto, Japan) and two ESA model 580 solvent delivery modules (ESA, Chelmsford, MA, USA). A Triathlon autoinjector (Spark Holland, Emmer, The Netherlands) with two six-port switching valves (Rheodyne, Cotati, CA, USA) was used with a 200- or 2000-μl loop (plasma or saliva analysis respectively). An SPD-10A UV detector from Shimadzu was used for

signal detection. These signals were stored and analysed using Powerchrom software (AdIstruments, Hastings, UK) running on an Apple Macintosh Performa 475 computer (Apple, USA). A Centra CL2 (International Equipment Company, Needham Heights, MA, USA) was used for sample centrifugation prior to injection. The post-column reaction took place in a 5-m knitted Teflon coil, with an inner diameter of 0.46 mm (Coricon, Knivsta, Sweden) immersed in a water bath (70°C) from Grant Instruments (Barrington, Cambridge, UK).

2.3. Chromatographic conditions

For sample preparation, an ADS-C_{18} column (Merck, Darmstadt, Germany) was used. The subsequent separation was performed on a reversed-phase C_{18} column (100×4.6 mm; 3 μm) protected by an R2 guard column, both from Chrompack (Bergen op Zoom, The Netherlands). An in-line filter (Merck) was placed in front of the RAM and guard columns, respectively.

2.4. Plasma sample preparation

Blank heparinised human plasma, purchased from the blood bank (University Hospital, Uppsala, Sweden) was used for preparation of samples with the following artemisinin concentrations: 5, 10, 20, 1000 and 2000 ng/ml. Plasma samples were centrifuged at 8000 *g* for 7 min before injection.

2.4.1. Saliva sample preparation

Blank saliva was collected from volunteers and pooled prior to preparation of artemisinin samples. The saliva was used for preparation of samples with a concentration range of 2–240 ng/ml. Saliva samples were centrifuged at 8000 *g* for 7 min before injection.

2.5. Column switching procedure

Fig. 2 shows a schematic diagram of the switching-system's set-up. Four steps (A–D) characterised the analysis of each sample:

(A) Sample application. Each sample was injected

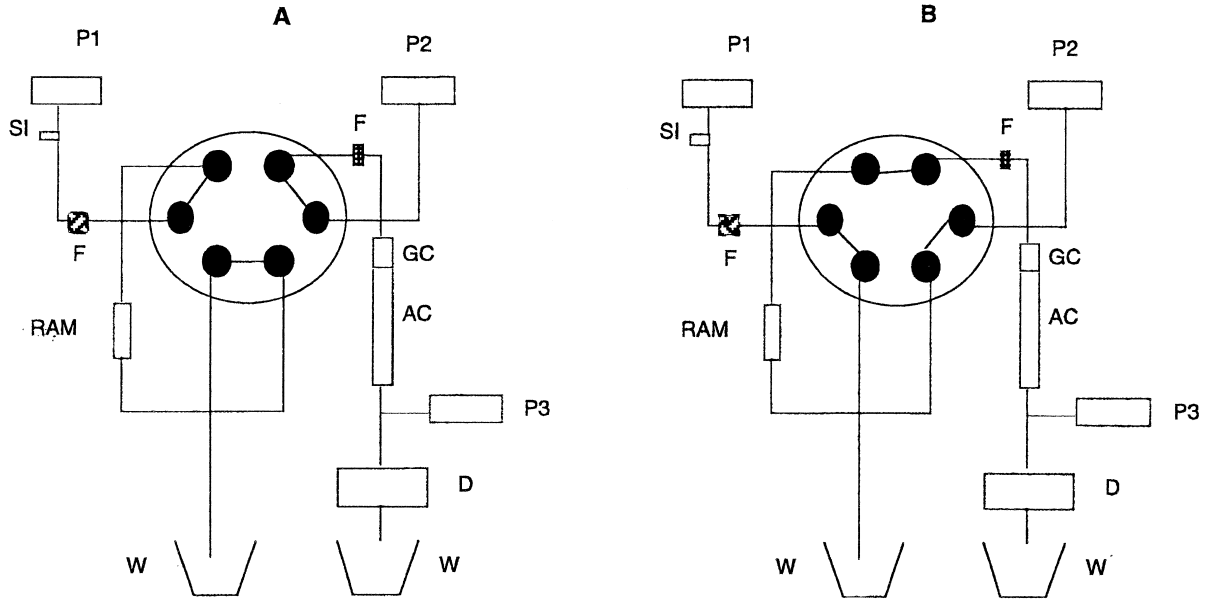

Fig. 2. Schematic presentation of the column-switching system: A, inject position and B, load position. Abbreviations: AC, analytical column; D, detector; F, filter; GC, guard column; P1 and P2, pumps 1 and 2, respectively; P3, post-column derivatization pump (KOH); RAM, restricted access material column; SI, sample injection and W, waste.

onto the RAM column with a mobile phase consisting of water–acetonitrile (98:2, v/v). This low acetonitrile content was chosen to avoid precipitation of proteins in the mobile phase [29]. Two-percent acetonitrile was found to be suitable, providing an adequate clean-up of the sample. The flow-rate of this mobile phase increased gradually from the initial value of 1.20 to 3.00 ml/min within 2 min after sample injection.

(B) Transfer. Nine min after the sample injection, the flow-rate was reduced to 1.20 ml/min and the first valve switching took place. The separation mobile phase (water–acetonitrile; 50:50, v/v) washed the RAM column in a back-flush mode with a flow-rate of 0.75 ml/min, transferring the retained artemisinin to the analytical column.

(C) Reconditioning. The valve was switched back to its initial position (position A) after 1.5 min, allowing the RAM column to equilibrate with the first mobile phase for 4.5 min before the injection of the next sample.

(D) Separation, derivatization and detection. After separation on the analytical column, the eluent was allowed to react with 0.3 *M* potassium hydroxide solution in an ethanol–water mixture (9:1, v/v) in the on-line Teflon coil immersed in a 70°C water bath. The presence of the derivatization product was detected by the UV detector, which was set at a wavelength of 289 nm.

2.6. Clinical application

Plasma and saliva samples were obtained from four consenting healthy male adult Vietnamese subjects who participated in a Phase I clinical drug trial to be reported separately. Capillary blood was sampled by lancing a fingertip (Microtainer lancet, 2.2 mm depth, Becton Dickinson, NJ, USA) and collecting into a microtainer Li–heparin tube, 400 μl (Becton Dickinson). Samples were left at room temperature for 5 min before centrifugation at 3500 *g* followed by separation of the plasma, which was frozen immediately. Unstimulated saliva was collected directly into cryotubes (Nunc, Denmark), immediately frozen and transported in dry ice to Uppsala for analysis. Injection volumes of the thawed plasma and saliva samples were 100 and 1000 μl, respectively.

3. Results and discussion

3.1. Selection of mobile phases

There are two major factors to consider when deciding the content of mobile phases to be used in the system: the choice and content of the organic modifier and the pH of the mobile phase. It is also of importance to match the two mobile phases thereby avoiding interfering system peaks. In our system, we chose acetonitrile as the organic modifier in both mobile phases (2 and 50%, respectively). Acetonitrile was chosen since the post-column derivatization system has already been shown to function properly [13]. During system development, methanol was tested in both mobile phases. However, since it offered no advantage compared to acetonitrile, the latter was chosen.

Artemisinin is an aprotic compound and, therefore, the pH of the mobile phase does not affect its retention behaviour. However, to maintain reproducible retention behaviour of protolytic compounds in the matrix and to maintain proteins and other endogenous compounds in their native states, it could still be desirable to include a buffer system in the mobile phase. A phosphate buffer, pH 7.5 (ionic strength, 0.05) was evaluated in the study. Addition of the buffer resulted in higher baseline noise without any detectable improvement of the chromatograms. Therefore, water was used for both mobile phases.

3.2. Selection of time-event for column-switching

In its initial position, valve position A (Fig. 2), the sample was loaded onto the RAM column. The switch to position B should occur when all proteins in the matrix have been eluted. It has been shown that more than 93% of the proteins in plasma can be recovered within 3 min for an ADS column using 5% isopropanol in water [30]. However, a longer time of 9 min was chosen as this resulted in improved stability of both the RAM and analytical columns.

All artemisinin was recovered in less than 1 min from the RAM column when eluted with the high-acetonitrile-content mobile phase. The valve was therefore kept in position B for 1.5 min before switching back to position A for re-equilibration with

the mobile phase containing 2% acetonitrile prior to the next sample injection.

Since clean-up and separation steps of two different samples can take place simultaneously, a total analysis time of 15 min for each sample could be achieved with artemisinin retention on the analytical column of 9 min after the switch to position B (19 min after sample injection).

3.3. Flow-rates

3.3.1. RAM column

During method development, the flow-rate of the weak mobile phase introducing the biological matrix to the RAM column was set to 1.00 ml/min. However, analysing a larger number of saliva samples resulted in an increased back pressure of the analytical column, followed by peak broadening. Visual examination revealed darkening of the guard column after 10 to 15 samples, suggesting that a 1.00-ml/min mobile phase flow-rate did not suffice to elute all endogenous compounds in a 1.00-ml saliva sample. Since further experiments showed that no artemisinin was lost at a flow-rate of 3.00 ml/min for more than 50 min, this flow-rate, with a duration of 9 min, was chosen. Under these new conditions, more than 200 samples could be analysed without any noticeable change in the performance of the analytical column.

3.3.2. Analytical column

A flow-rate of 0.75 ml/min of the second mobile phase resulted in a fast elution of artemisinin from the RAM column and an analysis time of 9 min on the separation column.

3.4. Post-column derivatization optimization

The post-column derivatization step was originally developed and optimized for the quantitation of artesunate, an artemisinin derivative [11]. In order to achieve the highest possible sensitivity for artemisinin, the optimal concentration of potassium hydroxide was investigated. It was found that a 0.3-*M* solution of the base in ethanol–water (9:1, v/v) at a flow-rate of 0.45 ml/min for the post-column derivatization solution gave the best results, aiming at maximizing the artemisinin peak area. Fig. 3 shows typical chromatograms of a plasma and a saliva sample.

3.5. Calibration and validation

Three different artemisinin stock solutions were prepared separately, two of which were used for the preparation of standard-curve samples (2 and 220 ng/ml for quantitation in saliva; 10 and 2000 ng/ml for plasma) and the third for quality control samples (5, 65, 129 ng/ml for saliva; 25, 402 and 1609 ng/ml plasma). Calibration curves were constructed by analysing eight standard samples, four each at the high and low levels, with half of the samples derived from each of the two stock solutions. It had previously been determined that the system response was linear within the studied concentration ranges. Calibration curves for each matrix were evaluated by ordinary least-squares regression.

Accuracy and repeatability were estimated by comparison of predicted concentrations versus actual concentrations. The lower limit of quantification (LOQ) of artemisinin was assessed by pre-made plasma/saliva samples. The LOQ was defined as the lowest concentration determined with a within-day coefficient of variation less than or equal to 20% and with an accuracy of between 95 and 105%, and was taken to be 2 and 10 ng/ml for saliva and plasma matrices, respectively (Table 1).

Quality control samples, analysed in duplicate on separate occasions, demonstrated acceptable inter-day variability of the system (Table 2).

Since direct sample injection eliminates the loss of drug due to extraction procedures, absolute recovery was not calculated. The relative recovery (matrix vs. water) was estimated by comparison of regression slopes as well as relative differences in the slope for detector response after injection of standard samples prepared from the same artemisinin stock solution in plasma, saliva and water, respectively. Slopes of the plasma and water calibration curves in the range of 10 to 2000 ng/ml (injection volumes of 100 μl) were 0.023 and 0.024, respectively, resulting in a relative deviation [(slope plasma−slope water)/slope water] of 4%. Slopes of the saliva and water standard

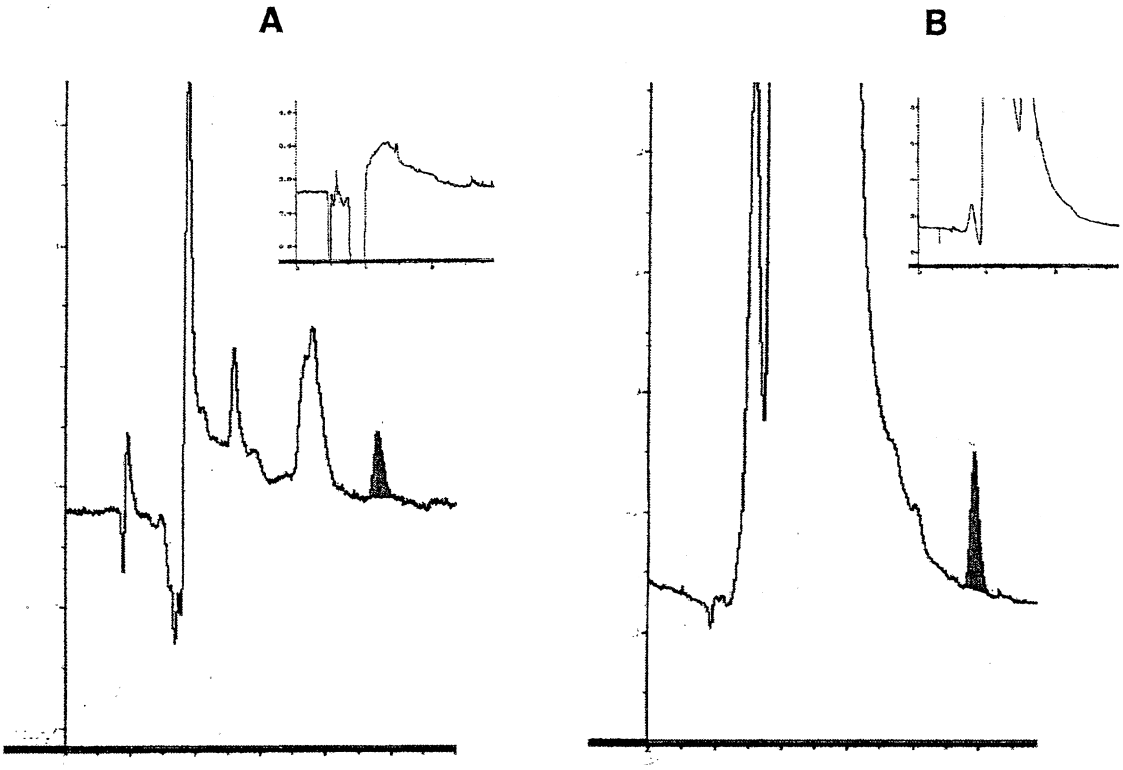

Fig. 3. Typical chromatograms after injection of spiked samples of artemisinin. (A): Plasma (100 μl) at a concentration of 20 ng/ml and (B): 1.0 ml of saliva containing 5.3 ng artemisinin/ml. Insets depict chromatograms after injection of blank plasma and saliva, respectively. The duration of each chromatography procedure was 10 min.

curves in the range of 2 to 220 ng/ml (injection volumes of 1.00 ml) were 3.993 and 4.165, respectively, giving a relative deviation of 4%.

3.6. Clinical application

Artemisinin concentrations in saliva and plasma samples from four healthy subjects are depicted in Fig. 4. Saliva concentrations were on average 0.23% (95% confidence interval: 0.18–0.27) of those in plasma sampled at approximately the same time points.

3.7. Chromatographic interference

With combination chemotherapy increasingly being advocated for the treatment of malaria [31,32], the chromatographic interference between artemisinin and several antimalarial drugs and their main metabolites was tested. None of the tested

Table 1
Within-day accuracy and precision for quantification of artemisinin in plasma and saliva samples at different concentrations.

Matrix	Concentration (ng/ml)	Found concentration (mean±SD), (ng/ml)	CV (%)	Accuracy (%)
Plasma	10.1 (n=8)	9.96±9.05	10.5	98.6
	20.1 (n=8)	20.8 ±0.83	4.0	103.4
	2014 (n=6)	2014±20.6	1.0	100.0
Saliva	2.0 (n=8)	1.97±0.22	11.1	98.6
	2314 (n=8)	231.4±7.0	3.0	100.0

Table 2
Between-day precision for artemisinin quantified in plasma ($n=6$) and saliva ($n=6$) samples, respectively

Matrix	Concentration (ng/ml)	Between-day variation (CV%)
Plasma	24.94 ($n=6$)	15.8
	402.3 ($n=6$)	10.2
	1609.2 ($n=6$)	8.6
Saliva	5.3 ($n=6$)	10.17
	64.5 ($n=6$)	11.79
	129.0 ($n=6$)	8.63

compounds (mefloquine, chloroquine, deacetyl-chloroquine, sulfodoxine, acetylsulfodoxine, pyrimetamine, proguanil, cycloguanil and 4-chlorophenylbiguanide) showed any interference when co-injected with artemisinin in an aqueous solution. The interference of other drugs and metabolites cannot be ruled out.

3.8. Column stability

More than 300 plasma and 400 saliva samples (100 μl and 1.0 ml each, respectively) could be analysed without any noticeable change in the performance of the RAM-column. However, change of the in-line filters after 200 samples was necessary to prolong the column's lifetime.

In our experience, the analytical column was the most vulnerable component in the system. Despite a high flow-rate through the RAM-columns, there was still a need to change the analytical column after approximately 400 saliva samples. A more-frequent change of the guard column (after 200 samples) could extend the lifetime of the analytical column.

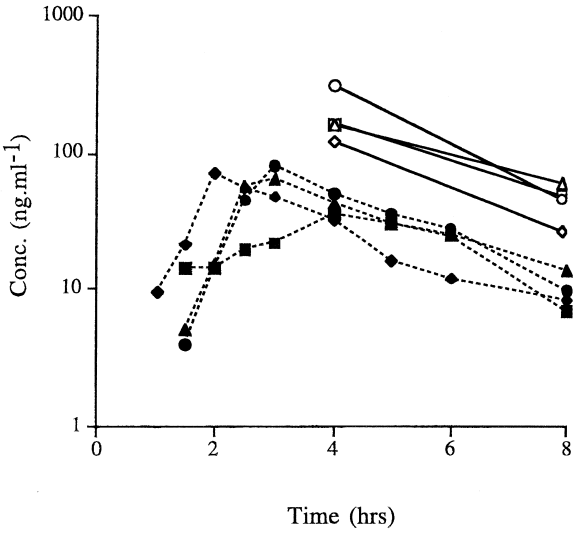

Fig. 4. Artemisinin concentration determined by direct injection of saliva (closed symbols) and plasma from capillary blood samples (open symbols) obtained from four healthy Vietnamese subjects (○, □, △, ◊) after oral administration of 500 mg of artemisinin, with on-line sample clean-up on a RAM column coupled to a HPLC system with on-line post-column alkali derivatisation with UV detection.

4. Conclusions

The presented coupled column HPLC system employing a restricted access material column offers a substantial decrease in time and labour for the routine analysis of artemisinin in plasma and saliva. Moreover, such an automation should contribute to fewer errors compared with off-line clean-up systems by reducing the steps in the analytical procedure.

In terms of quantitation limits, the method is comparable with other systems reported but with the added advantage that total plasma volumes as low as 110–130 μ1 are quantified instead of the 0.5 to 1.0 ml required in other current methods. This method is therefore particularly suited for the quantitation of artemisinin in capillary blood samples. Importantly, the sampling of capillary blood for drug quantitation simplifies and enhances pharmacokinetic investigations of antimalarials such as artemisinin in non-hospitalised, and pediatric in particular, patient populations. Obtaining saliva samples is an attractive alternative since samples can be obtained more frequently but it places higher demands on assay sensitivity since only unbound artemisinin is being measured. It being possible to inject larger volumes of saliva onto the RAM column, a quantitation limit as low as 2 ng/ml was achieved, which should be acceptable for use of this matrix in clinical pharmacokinetic studies.

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