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Rapid determination of artemisinin and related analogues using high-performance liquid chromatography and an evaporative light scattering detector

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Abstract

Artemisinin and its analogues are a class of compounds of current interest in the treatment of drug-resistant malaria. These antimalarials are preferentially taken up into malaria infected erythrocytes as compared to uninfected erythrocytes, a fact that may represent an important parameter in drug potency. Numerous methods for the analysis of specific artemisinin analogues have been developed, but most are not widely adaptable to a large range of analogues. In this paper we describe a high-performance liquid chromatographic method developed and validated for artemisinin and several analogues of artemisinin using a readily available evaporative light scattering detector. This quantitation method was found to be straight forward, rapid, inexpensive and reproducible. Standard calibration curves constructed for six artemisinin compounds were linear with the detection limit determined between 6 and 60 ng. The intra- and inter-day accuracy were found to be 2.75% and 4.15%, respectively with less than 3% variation in precision. The validated assay was applied to a mixture of artemisinin derivatives, where they were easily separated and quantitated.

Keywords: Artemisinin

1. Introduction

1.1. Artemisinins

It is estimated that one million children die from cerebral malaria and related complications such as anemia and kidney failure in Africa each year [1]. Most fatalities from malaria occur south of the Sahara Desert in Africa, but other high risk areas are in Brazil, India, Sri Lanka, Thailand and Vietnam. People most at risk of dying from malaria are non-immunes (children), pregnant women, workers moving from low to high areas of malaria transmission, and particularly, people from industrialized, non-malarious parts of the world who catch malaria and return home. The severe and complicated stages of malaria have a mortality rate of between 20 and 50% and the parasite responsible for most fatal malarial infections, *Plasmodium falciparum*, is capable of causing demise in patients in a matter of a few hours

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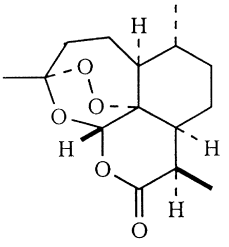
[2,3]. While there are still areas in which conventional antimalarials such as chloroquine, mefloquine, or pyrimethamine–sulfadoxine combinations are effective against malaria, those areas are by far on the decline due to the development of drug resistance. Interest in the development of new antimalarial drugs acting by novel modes of action is fostered by the widespread occurrence of multi-drug resistant *Plasmodium falciparum* (human malaria).

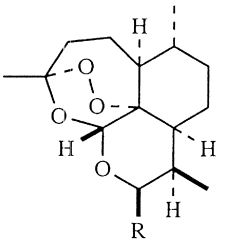
The Artemisinin class of drugs is considered to be the most promising compounds in the search for treatment of drug-resistant malaria. These sesquiterpene antimalarial compounds possess an endoperoxide bridge (C–O–O–C), as represented by the parent drug artemisinin [4] (Fig. 1). Artemisinin itself is a naturally occurring compound found in the leafy portions of the herb, wormwood, or *Artemisia annua L.* and has been used to cure fevers in China since 168 B.C.

Dihydroartemisinin [5] (Fig. 1), a first generation analogue and metabolite of artemisinin, is known to be more therapeutically active than artemisinin and is responsible for the antimalarial action of some of the analogues following in vivo metabolism. Oil soluble analogues such as arteether [6–8], artemether [6–10], and water-soluble analogues such as sodium artesunate [11–13], (Fig. 1) have also been synthesized to obtain better bioavailability of the drug. In recent years simple analogues of artemisinin, such as artemether, have been clinically used in China for the treatment of multi-drug resistant malaria.

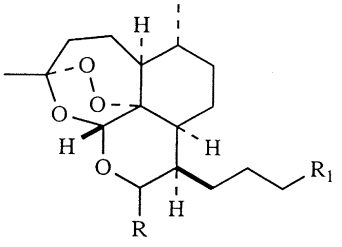
Even though over one hundred analogues of the natural product as well as radiolabeled artemisinin have been synthesized, information concerning the



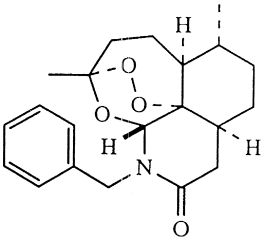
1, Artemisinin



2, R = OH, dihydroartemisinin
3, R = OEt, arteether
4, R = OMe, artemether
5, R = $OOCCH_2CH_2CO_2Na$, sodium artesunate
6, R = H, 10-deoxoartemisinin



7, R = H, R_1 = 4-ClC_6H_4
8, R = H, R_1 = C_6H_5
9, R = H, R_1 = CH_3
10, R = OH, R_1 = C_6H_5



11

Fig. 1. Structures of artemisinin analogues.

red blood cell (RBC) uptake of artemisinins with regard to structure has not been reported. The artemisinin class of antimalarials are preferentially taken up into malaria infected erythrocytes as compared to uninfected RBCs [14–16]. The uptake of artemisinin and artemisinin analogues into infected RBCs may be an important parameter in drug potency and is therefore worthy of investigation. Although numerous methods for the analysis of specific artemisinin analogues have been developed, most are not widely adaptable to the extensive range of analogues that need to be tested. This is due, in part, to the fact that most of the methods' development were designed for the phytochemical screening of crude plant extracts, and therefore lack both the sensitivity and the selectivity needed for clinical pharmacological studies.

1.2. Methods of analysis

Although artemisinin as well as most of the first generation analogs absorb in the ultraviolet (UV) region between 210 and 220 nm, the extinction coefficients of these molecules are poor. As a result, the standard UV detection methodology is ineffective in the quantitation of these compounds. For this reason, the detection of artemisinin has been accomplished by using pre- and post-column derivatization to convert artemisinin into a UV active compound that absorbs with a large extinction coefficient at longer wavelengths [10,17]. A major disadvantage of this method is that, while the structure of the derivatized compound detected can be inferred, the chemical reaction for its production is not likely to work well for many artemisinin analogues.

High-performance liquid chromatography (HPLC) employing various detection methods such as chemiluminescent, reductive electrochemical detection well as the use of a mass spectrometer are available for the quantitation of artemisinin. Chemiluminescent methodology reports a detection limit of 2.5 ng for artemisinin [18] with a large dynamic range. However, the detector response greatly diminishes for arteether and artemether rendering it unsuitable for the quantitation of analogues. Reductive electrochemical methodology is fairly sensitive, reporting detection limits of 1–10 ng for the analysis of artemisinin and a few select analogues [9,19,20]. This method requires that detection of the analytes occur in the absence of oxygen and the mobile phase be rigorously deoxygenated before and during the analysis. The instrument must also be maintained in a completely oxygen free condition and all lines and junctions have to be air tight. Because of these restrictions, the electrochemical method was found to be time consuming. Liquid chromatography followed by thermospray mass spectrometry (LC–TSP-MS) detection has been used to quantitate arteether and its metabolites with a detection limit of 0.2 ng [7]. While this method may work well for the identification and quantitation of the wide range of artemisinin analogues to be tested, the equipment is relatively expensive and may not be available in every laboratory.

One of the most sensitive methods developed is the radiolabeling of the artemisinin compounds using ^{3}H or ^{14}C isotopes [15,16,21]. Meshnick et al. have reported the in vitro uptake of three artemisinin analogues ([^{3}H]dihydroartemisinin, [^{3}H]arteether, and [^{14}C]arteflene) by *Plasmodium falciparum* infected RBCs [21]. Although the detection of radiolabeled compounds is extremely sensitive, it is very expensive, time consuming and requires specially trained personnel.

Other liquid chromatographic methods have been developed, such as, capillary and super critical fluid chromatography using flame ionization detection [22], thin-layer chromatography [23], and gas chromatography–mass spectrometry [24]. Cyclic voltammetry [25], capillary electrophoresis using low-wavelength UV detection [11,26], visual absorption spectrometry [27], proton magnetic resonance [28], immunodetection [29], and polarographic detection [30] have also been used. Titrimetric methods such as iodometric detection [31] and acid–base titration [32] have been developed to detect and quantitate artemisinin and its analogues. Although these methods work well for specific artemisinin compounds, we have not found them useful for the quantitation of the number of artemisinin compounds needed to perform a comprehensive uptake study. To adequately address these shortcomings, a HPLC method was developed using the relatively inexpensive evaporative light scattering detection (ELSD) method for the analysis of artemisinin and analogues. Analytical methods using ELSD have previously been de-

veloped in conjunction with liquid chromatography and supercritical fluid chromatography for analytes difficult to detect by traditional methods [33–36] thus, rendering it a suitable method to quantitate artemisinins.

1.3. Evaporative light scattering detector

The principle of operation of ELSD [37–39] includes the nebulization and evaporation of the mobile phase using a stream of nitrogen gas. The effluent from a chromatography column is first nebulized into fine droplets from which the solvent can be easily evaporated. These droplets then enter the nebulization chamber. In this chamber, a narrow droplet size distribution is created by eliminating the larger droplets which condense on the sides of the glass walls of the chamber and flow outside through a siphon-overflow. Evaporation occurs as the nebulized droplets are carried by the gas flow to the drift tube where the more volatile mobile phase is converted to a gas and the analyte remains as particles. Finally, the solute particles emerging from the drift tube enter the light cell where they pass through a polychromatic light beam. The amount of light scattered is measured by a photomultiplier tube and is directly proportional to the amount of analyte in the column effluent.

It has been reported that ELSD can be used with most solvents including water and is able to detect all types of analytes regardless of their molecular construction. Unlike electrochemical detection, ELSD is insensitive to the composition of the mobile phase creating flat baselines with solvent gradient programs that cover a wide range of solvent polarities [34,35].

The purpose of the present investigation was to develop and validate a quick, accurate, sensitive and yet easy to use analytical method to quantitate analogues of artemisinin. We describe here a method which we feel satisfies these criteria. The described method uses reversed-phase HPLC coupled to an ELSD to quantitate eight artemisinin analogues. Specifically, we focus on the development as well as the careful validation of the HPLC–ELSD method. Several analogues of artemisinin were chosen to evaluate the method's potential application in the quantitation of all artemisinin analogs and metabolites. We also illustrate the usefulness of the method by separating and quantitating a mixture of two artemisinin analogues. Details of the method and validation are presented.

2. Experimental

2.1. HPLC system

Initially the Waters chromatography system (Waters, Milford, MA, USA) was equipped with two 510 programmable pumps, a Rheodyne 7725I injector and a 486 tunable UV detector. An evaporative light scattering detector SEDEX Model 55 (S.E.D.E.R.E., France) was added to the HPLC system in series after the UV detector. A Phenomenex 250×4.6 mm, reversed-phase C_{18} IBO-SIL silica column, 5 μm particle size (Phenomenex, Torrance, CA, USA) was selected. All injections were performed using a 20-μl loop. The chromatographic system was controlled using the 2010 Millennium (Waters, Milford, MA, USA) chromatography manager software (version 2.10) loaded on a Digital Venturis computer (Digital, Maynard, MA, USA). After the flow-rate and the composition of the mobile phase was established the Waters UV detector was removed from the system and all subsequent studies were performed using only ELSD.

Prior to each run, the HPLC–ELSD system was allowed to warm up for 20–30 min and the pumps were primed using the protocol suggested by the manufacturer. Using freshly prepared mobile phase, the baseline was monitored until stable and the samples run.

2.1.1. Mobile phase preparation

The mobile phase was prepared by mixing HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ, USA) and nanopure water (Barnstead, Neuton, MA, USA) in a predetermined ratio then filtering it through a 0.2-μm nylon filter using a vacuum filtration assembly (Phenomenex, Torrance, CA, USA). Filtered solvents were degassed using a water vacuum assembly with gentle stirring using a magnetic stirrer (Lab-Line Instruments, Melrose Park, IL, USA) for about 5 min.

2.2. ELSD parameters

The ELSD parameters (temperature, pressure and gain) were varied in order to obtain minimum noise and maximum detection signal to optimize the detection limit of artemisinin. The detector's performance was periodically evaluated by executing the electronic noise, solvent noise, column noise and signal stability tests using the procedures recommended by the manufacturer. Nitrogen (University of Mississippi, University, MS, USA) was used as the driving gas for nebulization and the carrier gas for analyte transport. The carrier gas was passed through filter frits prior to entering the detector to assure the absence of stray particles introduced by the gas.

2.3. Calibration curve and standards

In a clean, dry 10-ml volumetric flask, a sample (approximately 10 mg) of artemisinin (Dr. Mitchell Avery, University of Mississippi) was accurately weighed and dissolved in HPLC grade methanol to make a stock solution. It should be noted that this stock solution was found to be stable for three days when stored at 4°C. Calibration standards were prepared by diluting the stock solution with methanol in appropriate quantities. Eight calibration standards were made at concentrations between the detection limit of artemisinin and the concentration at which saturation of the detector occurred. Three controls were prepared so as to lie in the lower, middle and upper regions of the calibration curve. A calibration curve was generated by plotting the logarithm of area under the curve versus the logarithm of concentration [39]. Regression analysis was performed on the data points using the Cricket Graph software Version 1.3.2 (Cricket Graph, Malvern, PA, USA). The equation of the regression line was used to quantitate control and unknown samples. Chromatographic parameters were also optimized for the eight selected artemisinin analogues (compounds 2, 3, 6–11 Fig. 1) (Dr. Mitchell Avery, University of Mississippi) tested using the same protocol [4–13,40–43].

2.4. Validation of the HPLC–ELSD method for artemisinin [44]

All the criteria for the validation of the HPLC–ELSD method were set at 5%.

2.4.1. Within replicate variation

The eight artemisinin calibration standards were freshly prepared and six replicate injections of each standard were made into the HPLC–ELSD system on seven consecutive days. The mean detector response was obtained by calculating the mean value for the area under the curve (obtained from the chromatogram) for each of the six injections. The standard deviation (SD) and relative standard deviation (RSD) were also determined for each set of controls.

2.4.2. Intra-day accuracy

The intra-day accuracy was evaluated by establishing a calibration curve using eight freshly prepared standards. After the calibration curve was generated, a set of three freshly prepared controls were run (in triplicate) three times (at 3.5-h intervals) on the same day. The concentrations of the controls were determined using the equation of the line calculated from the calibration curve. The percent deviation from the theoretical concentration was calculated and used as the parameter to evaluate intra-day accuracy.

2.4.3. Inter-day precision and accuracy

The inter-day precision was evaluated by generating calibration curves using freshly prepared standards on each of three days. The controls were freshly prepared the first day and used on the two subsequent days. The controls were quantitated using the equation of the line calculated from a new calibration curve constructed on each of the three days. The percent deviation from the theoretical value was calculated for each control on each of the three days and used to evaluate intra-day accuracy. The variation within replicate injections were also calculated and used to evaluate intra-day precision.

3. Results and discussion

3.1. Chromatography

A methanol–water (80:20, v/v) mobile phase resulted in an elution time of 5.4 min using a flow-rate of 1 ml/min for artemisinin (Fig. 2). The detector temperature was optimized at 32°C, carrier gas pressure at 2.2 bar and signal gain at 12. It was observed that at detector temperatures higher than

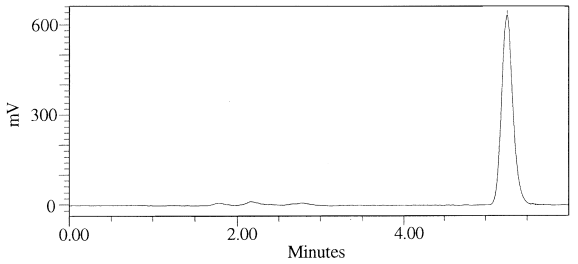


Fig. 2. Chromatogram of artemisinin. Mobile phase: methanol–water (80:20). Flow-rate: 1 ml/min. Injection volume: 20 ml. Detector temperature: 32°C. Carrier gas pressure: 2.2 bar. Signal gain: 12.

32°C the baseline noise increased; this may be attributed to improper evaporation of the nebulized eluate in the drift tube. It was also observed that the detector could not be operated properly at temperatures lower than 5°C below ambient temperature.

3.2. Validation of the HPLC–ELSD method for artemisinin

3.2.1. Limit of detection and dynamic range

A calibration curve was constructed for artemisinin and produced a linear dynamic range of 3 μg/ml to 70 μg/ml using the HPLC–ELSD method. The limit of detection (3 μg/ml or 60 ng/20 μl injection of artemisinin) was determined using the criteria of the lowest detectable amount above three-times the peak-to-peak baseline noise (3×p-p noise). Linear regression analysis of the log–log transformation of the area under the curve (for the calibration standards) verses the concentration of the calibration standards created an equation of the line equal to $y=3.99+1.67x$ with an $R^2=1.000$.

3.2.2. Variation within replicates (inter-day precision)

The RSDs between replicate injections ranged between 0.35% and 4.00% ($n=6$) over the seven days tested (Table 1). The higher RSD values were observed at the lowest concentration assayed, 7.5 μg/ml. As the concentration approaches that of the detection limit, baseline noise plays a role in the resolution and integration of the analyte peak resulting in an increase in the RSDs. This in turn causes the RSD among replicate injections to be high. The 4.00% ($n=6$) variation between replicates was well within the 5% criteria set.

3.2.3. Intra-day accuracy

Stability of the calibration curve within a given day was evaluated by injecting a set of three controls at three different times in a single day. It was observed that the standards were quantitated between −1.15% and +2.75% ($n=3$) of the actual value in each of the three sets (Table 2). The calibration curve was found stable for a minimum of 7 h based on a criterion of less than ±5% variation indicating stability.

3.2.4. Inter-day precision and accuracy

Variation within replicate injections (precision) of the controls on all four days were found to be within 2.82% ($n=3$) (Table 3). Also, quantitation of the controls (accuracy) fell between 4.15% and 2.66% ($n=3$) and did not to deviate with a bias on either the negative or the positive side. In general the errors in quantitation were found to be the highest in the control with the lowest concentration. Because the response of ELSD is sigmoidal and the calibration

Table 1
Relative standard deviations between replicate injections on seven days

Concentration (μg/ml)	RSD ($n=6$) (%)						
	1	2	3	4	5	6	7
7.5	2.03	1.52	2.00	3.93	4.00	3.64	3.87
10.0	0.95	1.58	2.35	1.65	3.06	2.05	4.67
15.0	2.84	1.81	1.33	2.82	2.19	1.41	2.07
20.0	2.08	2.28	1.30	1.21	1.01	2.53	1.29
25.0	1.95	2.32	1.48	2.03	1.82	1.30	1.95
30.0	1.40	3.71	3.27	1.21	1.96	1.38	1.79
35.0	1.40	1.47	3.11	2.11	2.69	0.35	0.60
40.0	2.70	1.68	0.51	0.88	2.23	0.61	1.31

Table 2
Determination of intra-day accuracy

Time (h)	Theoretical concentration (μg/ml)			Calculated concentration (μg/ml)			Deviation (%)		
	1	2	3	1	2	3	1	2	3
0	12.62	22.72	37.87	12.48	22.76	38.01	−1.15	0.15	0.36
3.5	12.62	22.72	37.87	12.79	22.63	37.93	1.31	−0.42	2.75
7	12.62	22.72	37.87	12.75	22.59	37.93	0.99	−0.59	0.15

Table 3
Inter-day precision and accuracy

Controls	Day 1			Day 2			Day 3		
	1	2	3	1	2	3	1	2	3
Theoretical concentration (μg/ml)	12.75	22.95	38.25	12.75	22.95	38.25	12.75	22.95	38.25
Calculated concentration (μg/ml)	13.09	23.73	37.98	12.22	23.03	37.61	12.47	22.75	38.28
Deviation[a] (%)	2.66	−3.39	0.17	−4.15	−0.35	−1.67	−2.19	−0.87	0.08
Variation within replicates[b]	1.15	2.82	2.35	2.71	2.48	1.12	1.88	1.23	0.91

[a] Accuracy.
[b] Precision.

curve is a log–log transformation of the data, there is a significant error in the quantitation of points at the lower and upper end of the curve. It can therefore be concluded that for optimum quantitation from the calibration curve, concentrations should be selected in such a way that they lie in approximately the mid region of the calibration curve when using the evaporative light scattering detector.

3.3. Calibration of select analogues

In order to determine if the developed HPLC–ELSD method is valid for other artemisinin analogues, eight analogues were studied. Chromatographic parameters were optimized to determine calibration and detection limits for five of eight analogues using the HPLC–ELSD method developed. For the other three analogues: arteether, 10-deoxyartemisinin [40], and compound 9 [41], detection limits using the UV detector were found to be better than for the ELSD system. This may be due to the analytes undergoing sublimation following nebulization in the detector. This phenomenon is being further investigated. The detection limits and chromatographic parameters for the five analogues calibrated using ELSD are given in Table 4.

The HPLC–ELSD method developed can be used to detect and quantitate some of the analogues with lower detection limits compared to artemisinin. The detection limits for compounds 7 [41] and 8 [41] are comparable; this may be due to their structural similarity as they differ only by the side chain

Table 4
Artemisinin analogues[a]

Compound	Detection limit (ng/injection)	Equation of the line	Correlation coefficient (R^2)
1	60 ELSD	$y=3.99+1.67x$	1.000
2	60 ELSD	$y=4.31+1.31x$	0.995
7	20 ELSD	$y=4.78+1.32x$	0.996
8	10 ELSD	$y=5.06+1.19x$	0.996
10	60 ELSD	$y=4.95+1.43x$	0.999
11	6 ELSD	$y=4.71+1.49x$	0.999

[a] Mobile phase: methanol–water (80:20). Flow-rate: 1 ml/min. Injection volume: 20 ml. Detector temperature: 32°C. Carrier gas pressure: 2.2 bar. Signal gain: 12.

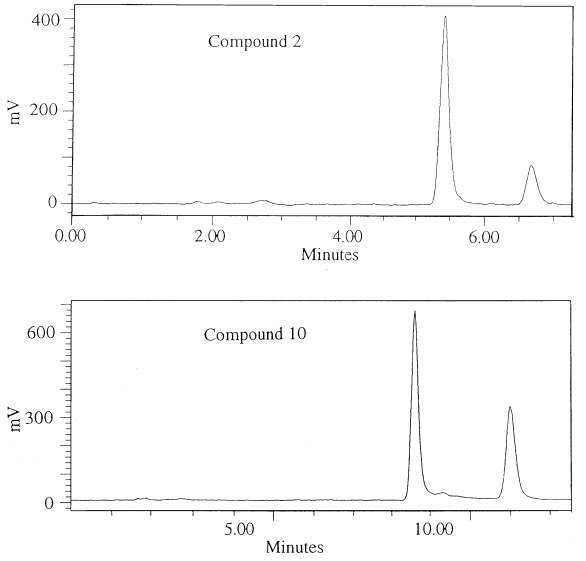


Fig. 3. Chromatograms of compounds 2 and 10. Mobile phase: methanol–water (80:20). Flow-rate: 1 ml/min. Injection volume: 20 ml. Detector temperature: 32°C. Carrier gas pressure: 2.2 bar. Signal gain: 12.

linkage. The chromatograms of compounds 2 and 10 (Fig. 3) show that there are two peaks present for each analyte that are due to diastereomerism and isomerism, respectively. Sandrenan et al. [45] reported that compound 2 (dihydroartemisinin) exists as two diastereomers (10α/10β) as shown in Fig. 3. The chromatogram of compound 10 [42] also shows two peaks which are due to the isomerism of the lactol moiety at C-10. In each case, the peak with greater area under the curve was used for calibration and quantitation of the analyte.

3.4. Separation and quantitation of a mixture

A mixture containing known concentrations of artemisinin and compound 7 was separated and quantitated (Fig. 4). It was found that the quantitation was 1.96% ($n=3$) deviated for artemisinin and 0.81% deviated for compound 7 from the respective known concentration (Table 5). From the results obtained it can be concluded that there is no interference between artemisinin and the analogue during the analysis. Although it may be rare for a sample to contain a mixture of artemisinins, the separation suggests that this method can potentially be used to separate and quantitate samples containing a mixture of artemisinin metabolites.

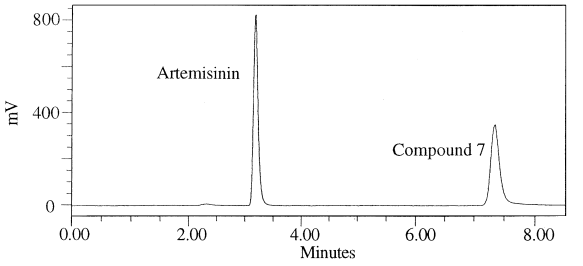


Fig. 4. Chromatogram of a mixture of artemisinin and compound 7. Mobile phase: methanol–water (90:10). Flow-rate: 1.25 ml/min. Injection volume: 20 ml. Detector temperature: 32°C. Carrier gas pressure: 2.2 bar. Signal gain: 12.

4. Conclusions

A high-performance liquid chromatographic method has been developed and validated for the detection and quantitation of artemisinin and select artemisinin analogues using an evaporative light scattering detector. With this method, artemisinin and eight artemisinin analogues were successfully quantitated, using a calibration curve, with detection limits of six of the nine compounds ranging between 0.3 and 3 μg/ml at 3×p-p noise. Although the detection limits for individual analogues were not as low as for some analytical methods reported, the HPLC–ELSD method described in this paper has the advantage of quantitating all of the analogues tested and most with reasonable detection limits. There were, however, three analogues tested that yielded poor detection limits which we believe was caused by sublimation of the compound in ELSD.

Validation of the HPLC–ELSD method for artemisinin and analogues included: inter-replicate variation, intra- and inter-day precision and accuracy. All of the validation parameters studied were found to have RSDs less than 5% and did not show a bias toward a single direction. This assay was successfully applied to a mixture of two artemisinin compounds. These compounds could be easily separated and quantitated from their respective calibration curves with an accuracy of 1.86% and 0.89% for artemisinin and the analogue, respectively.

Table 5
Separation and quantitation of a mixture of artemisinins

	Artemisinin (μg/ml)	Compound 7 (μg/ml)
Theoretical concentration	35.70	23.60
Calculated concentration	36.40	23.81
Deviation (%)	1.96	0.89

The HPLC–ELSD method was found to be rapid, relatively inexpensive, straight-forward and reproducible. Moreover, in principle it can be used to quantitate any artemisinin analogue of interest with little to no variation in the described method. There are a number of potential applications of this method such as the quantitation of artemisinin or artemisinin analogues in whole blood and blood products. A description of the use of this method for the quantitation of artemisinin analogues in RBCs will be the focus of a forthcoming article.

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