Development of HPLC analytical protocol for artemisinin quantification in plant materials and extracts

SUMMARY

Alexei A. Lapkin,[1] Adam Walker,[2] Neil Sullivan,[3] Bhupinder Khambay,[4] Benhilda Mlambo,[1] Smain Chemat[1]

[1] Centre for Sustainable Chemical Technologies, Department of Chemical Engineering, University of Bath, Bath, BA2 7AY, United Kingdom, A.Lapkin@bath.ac.uk
[2] Bioniqs Ltd., Biocentre, York Science Park, Heslington, York, YO10 5DG, United Kingdom
[3] SensaPharm Ltd, Unit 124i, BIC, Wearfield, SR5 2TA, Sunderland United Kingdom
[4] Kamtech Technologies Ltd, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom

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Abstract

Quantification of artemisinin purity and amount in plant material and extracts to date has been characterized by a considerable inconsistency in values. This is likely to be due to the adoption of varied analytical procedures and use of inappropriate to the specific applications analytical techniques. In this paper we are attempting to further develop artemisinin analysis to the point where a universally acceptable reference method is available to the research and end-users communities. Thus, we have developed and validated an HPLC-RI method and optimised an HPLC-ELSD method. We used the gradient HPLC-UV method recommended by the current artemisinin Monograph as a comparison for the method improvements presented herein, and show the limitations for its application scope. The data reported should help to allow more reliable laboratory analysis of artemisinin in both pure samples and in *A. annua* extracts.

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1. Introduction

A number of analytical procedures to quantify the amount of artemisinin in the collected biomass, in the primary and purified extracts, and to quantify its purity have been reviewed in the earlier literature [1-3]. Since these reviews a GC-MS method and principle component analysis for the analysis of the development of a metabolic profile of *A.annua* during different stages of growth was reported [4]. A proton NMR method of quantification was also proposed [5], although no validation data (limits of detection/quantification and precision) were reported. A modification to the LC-MS method was reported, establishing a very high sensitivity to artemisinin [6].

The WHO's monograph on *A. annua* cultivation [7] and the artemisinin monograph by the International Pharmacopeia [8] contain descriptions of several analytical methods. The simplest method is based on thin layer chromatography (TLC). Although TLC is most frequently used as a qualitative tool, quantitative and preparative TLC methods are available [9]. It is also widely accepted now that TLC systematically underestimates the amount of artemisinin in comparison to HPLC methods.

The most common method for analysis of artemisinin is based on high performance liquid chromatography (HPLC). The monographs [7, 8] describe an HPLC method using UV detection at 214 nm and dihydroartemisinin (5) as an internal standard, see Table 1. This method is validated only for the pure artemisinin and we claim in this work that it is not suitable for quantification of artemisinin in extracts.

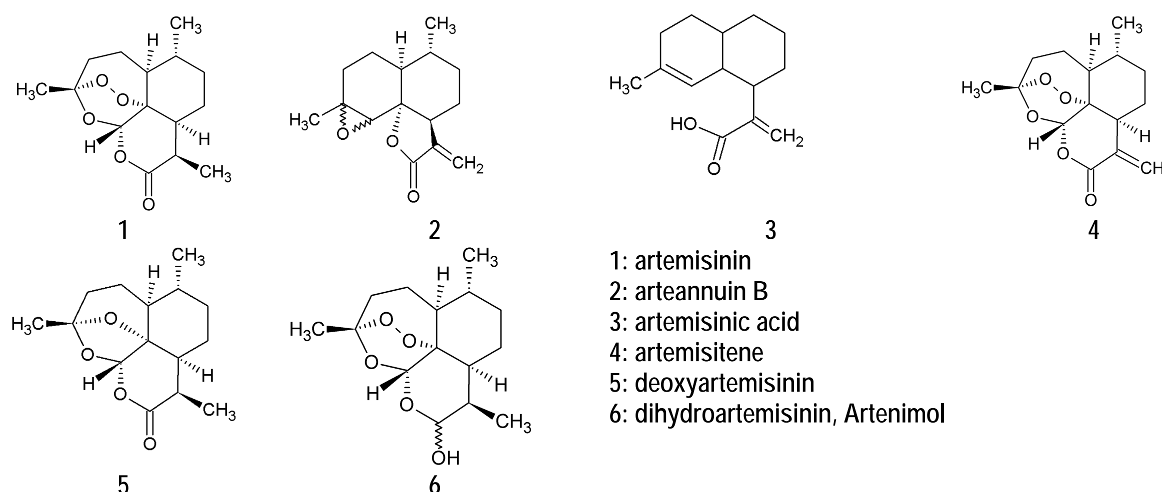
Many authors discard the WHO HPLC method on the basis of very low UV absorbance of artemisinin [1, 2, 10-13]. It was hence suggested to use pre- or post-column hydrolysis of artemisinin into more UV-active compounds to allow the use of the most wide-spread HPLC-UV instruments [11, 13, 14]. There are also reports on using other detectors, such as electrochemical reduction [15, 16] and evaporative light scattering (ELSD) [12, 17]. However, the issues surrounding accurate determination of artemisinin quantity will not be resolved by simply replacing the detector on an LC system.

The purpose of this study was to systematically address the issue of HPLC methodology for quantification of artemisinin in plant material and extracts.

Table 1. The conditions for HPLC analysis based on the International Pharmacopeia artemisinin monograph [8].

Time / min	Acetonitrile / % v/v	Water % v/v	Comment
0-17	60	40	Isocratic
17-30	60 → 100	40 → 0	Linear gradient
30-35	100 → 60	0 → 40	Return to initial conditions
35-45	60	40	Isocratic re-equilibration

The column is 100 x 4.6 mm with a 3 μm particle stationary phase (*the nature of the stationary phase is not specified in the Monograph*). The recommended flow rate of the mobile phase is 0.6 mL min^{-1} and detection is by UV at 216 nm.



Scheme 1. Chemical structures of artemisinin, some co-metabolites and important impurities

2. HPLC Stationary Phase Optimization

Sixteen different columns of 150 mm and 250 mm length based on C18 and other stationary phases were tested. Separation of artemisinin from deoxyartemisinin, artemisinic acid, artemisitene and dihydroartemisinin, as well as separation of artemisinin peak from other components in the extracts were studied.

Instrumentation

A Gilson HPLC instrument equipped with a UV (Spectromonitor 3000 LDC/Milton Roy, set at 220 nm) and ELSD (PL-EMD 960 by Polymer Laboratories) detectors in line was used. ELSD nitrogen flowrate was 3.6 L min^{-1} and evaporation temperature 55 ºC.

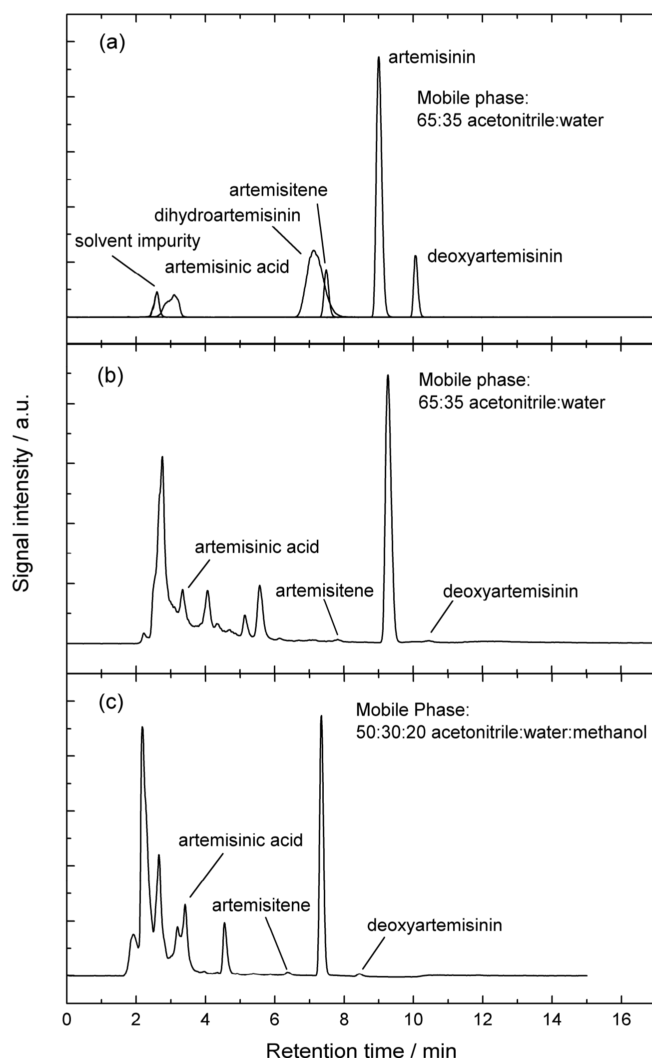
Best separation, especially for the case of deoxyartemisinin – artemisinin, was achieved on columns with aromatic groups bonded to the stationary phase. However they are inferior to standard C18 columns for profiling plant extracts. C18 columns differed in performance between manufacturers. The 250 mm columns were superior in performance to the 150 mm columns for the same stationary phase. The better columns were: Luna 5μ C18 250 x 4.6 mm (Phenomenex) and Betasil C18 5 μm 250 x 4.6 mm (Thermo Fisher Scientific).

3. HPLC Mobile Phase Optimization

Six different mobile phases were screened and their performance in terms of separation of main peaks, elution order and repeatability of retention time was analysed.

Instrumentation

This work was done on a Gilson instrument described above and using a Shimadzu Prominence HPLC equipped with a UV-vis diode array (SPD-M20A, DAD) and *in line* evaporative light scattering (ELSD, LTII, 350 kPa N_2, nebulizer at 40 ºC) detectors. Columns used were: (1) Shimadzu XR-ODS 50 x 2 mm with a 2.2 μm deactivated type B silica, 12 nm pores at a column flow rate 0.5 mL min^{-1} and (2) a Betasil C18 5 μm 250 x 4.6 mm at column flow-rate 1.0 mL min^{-1}. The Shimadzu instrument is equipped with a column oven and column temperature of 45 ºC was used.



Two mobile phases were found to be most adequate and comparable in terms of repeatability of retention time and in terms of coefficient of variation of the artemisinin concentration:

(1) A combination of acetonitrile, water and methanol (50:30:20 % v/v) was found to be the most effective for the HPLC analysis of *A. annua* extracts, giving better peak shapes and resolution of artemisinin from impurities. This mixture also allows a wider range of compounds to be dissolved in the injection solvent so that profiling is more representative of the extracts.
(2) Also, acetonitrile:water (65:35 % v/v) mobile phase produced adequate performance.

Both mobile phases were compared with the same column, Betasil C18 250 x 4.6 mm, and produced comparable results. There is a slight shift in retention time of artemisinin peak and positions of some of the by-products peaks in the traces of the extracts. However, good baseline separation and good precision was attained with both mobile phases. The addition of methanol produces somewhat sharper peaks.

Figure 1. Comparison of HPLC-ELSD traces obtained using two different mobile phase systems on a Betasil C18 250 x 4.6 mm column:

(a) standards dissolved in acetonitrile;
(b) acetonitrile:water 65:35 % v/v;
(c) acetonitrile:water:methanol 50:30:20 % v/v.

The concentrations of standards used: artemisinin: 1.85 mg mL^{-1}; artemisinic acid: 0.60 mg mL^{-1}, artemisitene: 0.89 mg mL^{-1}, dihydroartemisinin: 1.70 mg mL^{-1}, deoxyartemisinin: 1.42 mg mL^{-1}.

4. HPLC-RI Method Validation

Instrumentation

A Dionex Ultimate 3000 HPLC instrument equipped with an RI detector (Shodex RI-101) was used to develop a new protocol based on refractive index detection. A Phenomenex Gemini 5 μm C18 11 nm 250 x 4.6 mm column with a guard column was used. The mobile phase was 60:40 (% v:v) acetonitrile:water at 1.0 mL min^{-1} at ambient column temperature.

For development of the HPLC-RI method chloroform extracts were used. Chloroform *A. annua* extracts were prepared from 3 g dried plant tissue extracted with 80 mL chloroform for 10 minutes with stirring at room temperature. The extract was stripped of solvent *in vacuo* and re-dissolved in 5 mL mobile phase. An equal volume of internal standard (β-artemether prepared in mobile phase at 2.5 mg mL^{-1}) was added to the extract. Samples were filtered through a 0.2 μm syringe filter before injecting onto the column. A 100 μL injection loop was used for this study.

The inter day precision was found to deteriorate considerably for low concentration of extracts. This is attributed to degradation of artemisinin in solutions. A more detailed study of artemisinin degradation in different solvents will be undertaken during 2009 (contact Malcolm Cutler of FSC Development Services Ltd for more details).

Table 2. Summary of characteristics of the HPLC-RI method of artemisinin detection and quantification.

Linearity / R^2	Repeatability, CV / %	Limit of detection / mg mL^{-1}	Limit of quantification / mg mL^{-1}
0.9995	6	0.025	0.1

5. Comparison of gradient HPLC-UV method with isocratic HPLC-ELSD, HPLC-UV and HPLC-RI methods

A direct comparison between UV and ELSD methods of detection was made using acetonitrile dissolved artemisinin standard solution. In these experiments we used two mobile phases, containing (i) acetonitrile:water:methanol (50:30:20 % v/v), and (ii) acetonitrile:water (65:35 % v/v). See results in Tables 3 and 4.

Table 3. Comparison of linearity and limits of detection and quantification of UV and ELSD detection of artemisinin.

Method	Linearity over 0.1 – 10 mg mL^{-1}		Limit of detection		Limit of quantification	
	R	R^2	mg mL^{-1}	CV / %	mg mL^{-1}	CV / %
Gradient UV[a], 213 nm	0.9999	0.9999	0.009	6.7	0.020	10.0
Gradient ELSD[a]	0.9970	0.9950	0.001	9.2	0.100	3.1
Isocratic UV[b], 210 nm	0.9999	0.9999	0.001	0.25	0.009	0.7
Isocratic ELSD[b]	0.9990	0.9979	0.020	0.7	0.100	2.4

[a] Mobile phase: see Table 1.

[b] Mobile phase: 50:30:20 acetonitrile:water:methanol, %v/v.

Table 4. Comparison of intermediate precision of HPLC-UV (gradient), and HPLC-ELSD (isocratic) methods.

Mobile phase / method	Retention time CV / %		Repeatability of retention time CV / %	Concentration CV / %		Concentration accuracy / %	
	Intra day	Inter day		Intra day	Inter day	Intra day	Inter day
Gradient, see Table 1	0.54	0.87	0.47	0.16	0.27	99.4	98.8
Isocratic, 50:30:20 % v/v acetonitrile:water:methanol	0.06	0.40	0.04	0.25	7.6	96.0	110.0
Isocratic 65:35 % v/v acetonitrile:water	0.03	0.10	0.04	0.97	6.34	100.8	105.6

The UV detection calibration was found to be linear over a wide range of concentrations, at least within 0.1 – 10 mg mL^{-1}, whereas the ELSD detector is inherently non-linear, best fitted either by several linear relationships over narrow ranges of concentration, or by a polynomial or a power relationship over a broad range of concentrations. The linearity determined over 0.1 – 10 mg mL^{-1} range is better in the case of UV detection. The UV detector used also exhibited a better signal to noise characteristic, which allowed a lower limit of quantification in comparison with the ELSD detector.

The ELSD detection appears to show better characteristics when used in conjunction with the isocratic LC mode. The isocratic methods using the ELSD detector appear to have better repeatability of retention time, which is an important factor in the case of identification of artemisinin in the extracts. Between the two isocratic methods using different mobile phases, the mobile phase containing methanol gives a marginally worse interday precision and a wider concentration accuracy window. The UV-based gradient method gives the highest concentration precision and good accuracy for the concentrated standard solutions in comparison with the ELSD based isocratic methods.

The limit of quantification in the HPLC-RI method is similar to that of HPLC-ELSD method, however, a larger injection volume must be used (100 μL vs 20 μL).

The accuracy of the HPLC-UV and HPLC-ELSD methods does not compare well with the required purity of artemisinin by the current monograph, requiring that artemisinin content by HPLC-UV is within 97-102 % [8]. The gradient HPLC-UV method and the isocratic HPLC-ELSD method with acetonitrile:water (65:35 %v/v) mobile phase provide just enough accuracy for the monograph standard. However, in order to detect any impurities with a lower content, i.e. within the last ±2 %, a different, more sensitive method is required, for example, an LC-MS.

6. Conclusions and Recommendations

This study addressed the issues of methodology for sample preparation, optimization of HPLC conditions, selection of best detection techniques and the identification of some impurities in the analysis of artemisinin in extracts. This work is intended to contribute to standardization of procedures and ultimately to the development of a network of qualified laboratories capable of providing accurate and consistent quantification of this important natural product.

UV detection of artemisinin is shown to be a viable option. The UV method of detection has better accuracy and better limits of quantification in comparison to HPLC-ELSD. However care must be taken to make sure correct peak is identified and the LC method used allows for good baseline separation of the rather small artemisinin peak. In this respect, we do not recommend using HPLC-UV method for quantification of artemisinin in extracts, but only for determination of the purity of bulk artemisinin.

Clear separation of deoxyartemisinin and several co-metabolites of artemisinin is possible, including quantification. However, readily available standards are required and are currently not available. It was shown in this study that both UV and ELSD detectors miss some of the compounds in the extracts, which can be picked up by mass spectrometry. The importance and persistence of these compounds in consecutive processing of artemisinin is yet to be determined. Although detection of some key impurities, such as deoxyartemisinin, is possible with the HPLC-ELSD method, the accuracy of all HPLC methods is insufficient to improve on the current monograph's purity standard of 100±2 %.

The HPLC-RI method has been extensively validated and showed a similar limit of quantification, in terms of concentration, to that of HPLC-ELSD method. However, a larger sample loop is required for the HPLC-RI method, due to the lower absolute sensitivity of this detector.

The obtained results on exhaustive extraction of artemisinin from biomass for initial total amount determination show that the choice of solvent would affect the values, as would the procedures for extraction and for sample preparation. Acetonitrile and ethyl acetate were shown to be the best solvents for this purpose.

Overall, the HPLC-ELSD method appear to be the most robust for routine quantification of artemisinin in plant extracts, either for the purpose of quantification of artemisinin content in the leaf, or for optimization of extraction/purification protocols. The HPLC-UV method is not recommended for the analysis of extracts, but only for the analysis of the purity of bulk artemisinin. Analysis of impurities in artemisinin beyond the level set by the current Monograph requires the use of MS detection, due to its much higher sensitivity.

Based on the experiences gathered during this study we can recommend the following methods:

1) For analysis of artemisinin content in the leaf.
   Use acetonitrile or ethyl acetate as a solvent. Exhaustive extraction from plant material must be confirmed by sequential extraction to quantify the residual amount in the leaf after first extraction. Stability of artemisinin in the extract must be taken into account. Extracts should be analysed immediately without prolonged storage. Use HPLC-ELSD method for quantification of artemisinin in the leaf on the basis of exhaustive extraction. If only UV detector is available, retention time of the artemisinin peak must be confirmed on the same day when extract is being analysed. Use 210-216 nm wavelength of UV detector. C18 column of 250 mm length, e.g. Betasil C18 5 μm 250 x 4.6 mm at column flow-rate 1.0 mL min^{-1} gives consistently good results. Best mobile phases for analysis of extracts: (i) acetonitrile:water 65:35 % v/v; (ii) acetonitrile:water:methanol 50:30:20 % v/v.
2) For analysis of impurities in artemisinin and its purity to the level of the current monograph.
   HPLC-ELSD method with correct column and mobile phase allows identification of some impurities. HPLC-UV method allows to check purity of artemisinin to the level of current Monograph.
3) For analysis of purity and impurities profile beyond the level of the current Monograph: LC-MS and LC-MS-MS methods must be used.

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