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Keywords:

Capillary supercritical fluid chromatography, Packed supercritical fluid chromatography, Evaporative light scattering detector, Drug analysis, Plant extract

Summary:

The analysis of an antimalaric compound and its bioprecursor present in a herb (Artemisia annua L.) was carried out by supercritical fluid chromatography (SFC). Capillary and packed columns coupled respectively with a flame ionization detector (FID) and an evaporative light scattering detector (ELSD) were used. Both methods have been optimized in order to separate artemisinin and artemisinic acid. In comparison to conventional gas and liquid chromatography, SFC methods can be used to determine directly the two compounds (Without degradation and/or derivatization) in the concentration range of interest. Results obtained with plant extracts (following a supercritical fluid extraction) by capillary SFC-FID were confirmed by GC-MS.

1.INTRODUCTION

Artemisinin, an endoperoxide-containing sesquiterpene lactone, is known to be an efficient drug against chloroquine-resistant strains of *Plasmodium falciparum* parasites and cerebral malaria [1,2,3]. This compound is present in an herb, *Artemisia annua L.*, used for many centuries in Chinese traditional medicine. Even if total syntheses of artemisinin have been reported [4], the plant remains the only practical source of this compound. Hence, it is necessary to develop analytical methods able to detect artemisinin and its bioprecursor, artemisinic acid in the plant.

A lot of analytical methods have been published for the determination of artemisinin, such as HPLC [5,6] TLC [7], GC [8], ELISA [9], etc, but very few allow the simultaneous detection and quantification of artemisinin and derivatives.

In fact, artemisinin is thermolabile and can not be determined without a degradation process using GC. Furthermore, this compound is UV-transparent and requires a derivatization with NaOH before an HPLC-UV analysis. Therefore, we developed two supercritical fluid chromatography (SFC) methods. A capillary SFC coupled with a FID and a packed SFC coupled with an evaporative light scattering detector (ELSD). Both methods permit to separate and to quantitate artemisinin and artemisinic acid without derivatization and without decomposition. The capillary SFC procedure has been validated and compared with a conventional GC-MS. Finally, examples of application on real plant extracts obtained by supercritical fluid extraction will be presented.

2. MATERIALS AND METHODS

2.1 Materials

For supercritical fluid chromatography and extraction, 99.99% purity CO2 (Polygaz, Geneva, Switzerland) contained in a cylinder with an eductor tube was used. A charcoal packed column and a molecular sieve packed column were incorporated in front of the pump to prevent possible contamination by hydrocarbons.

HPLC grade methanol and acetonitrile were purchased from Maechler AG (Basel, Switzerland). Crystalline artemisinin was obtained from Sigma SA (SIGMA, St-Louis, USA), artemisinic acid was donated by Dr N. Acton (Walter Reed Army Institute of Research, Washington DC, USA), Dodecanol was from Merck (Darmstadt, Germany).

2.2 GC-MS analysis

GC-MS analyses were performed with a HP 5890 series II gas chromatograph coupled with a HP 5972 mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA). Injection was performed in the splitless mode (250°C) and the injection volume was 1 µl. Helium was used as the carrier gas at a flow rate of 1.6 ml/min. A 25 m x 0.2 mm I.D. HP-5 capillary column, film thickness 0.33 µm (Hewlett-Packard, Palo Alto, CA, USA) was used. The following GC temperature program was used: initial temperature 120°C, increased at 6°C/min to 250 °C, with a final hold for 4 min.

2.3 Capillary SFC analysis

SFC analyses were performed on a Carlo-Erba SFC 3000 with FID detection (Carlo Erba Instruments, Milano, Italy). Injection was performed in pneumatic actuation, the injection volume was 200 nl, the injection time was 1 s. The following capillary columns were used: A DB-WAX (polyethylene glycol phase) 20 m x 0.1 mm I.D., film thickness 0.1 µm (J&W Scientific, Folsom, USA); a DB-1701 (14% Cyanopropylphenyl-methylpolysiloxane phase) 20 m x 0.1 mm I.D., film thickness 0.1 µm (J&W Scientific, Folsom, USA); a DB-1 (Dimethylpolysiloxane phase) 20 m x 0.1 mm I.D., film thickness 0.1 µm (J&W Scientific, Folsom, USA). The following density program was used: initial density 0.25 g/ml hold for 7 min, increased at 0.04 [g/ml]/min to 0.75 g/ml, with a final hold for 7 min. The temperature of the oven was set isothermal at 100°C. The restriction was done by a 10 ml/min (at 0.25 g/ml) tappered fused silica restrictor (home-made). The detector temperature was 300°C.

2.4 Packed SFC analysis

The analyses were performed with a VARIAN 2510 HPLC pump (VARIAN, France) equipped with a cooling jacket for CO_2 and a polar modifier was added through a T Junction with a KNAUER HPLC pump 64 (KNAUER, Berlin, Germany). The supercritical fluid was homogenized by passing through a column filled with glass beeds. A Rheodyne 7125 injection valve with a 20 µl injection loop was used for sample introduction. The analyses were performed on a Nucleosil 50-5 column, 125 x 4 mm, and a Nucleosil 50 C 18 (MACHEREY-NAGEL, Oensingen, Switzerland) at a temperature of 40°C. The flow rate of CO_2 was set to 4.0 ml/min at a pressure of 250 bar.
The outlet of the column was introduced in the SEDEX 55 evaporative light scattering detector (S.E.D.E.R.E, France) through a pinched peek restrictor (home-made) heated at 80°C to avoid dry ice formation. The conditions of the ELSD were set as following: gas pressure 0.5 bar, temperatures of the nebulisation chamber and of the heating chamber were set at 40 °C. Integration was done by a HP 3396 series II integrator.

2.5 Supercritical fluid extraction

The air dried plant material was thoroughly ground in a domestic mixer. 100 mg of this material was extracted in a supercritical fluid extractor (Jasco LC-900, Jasco International Co. LTD., Tokyo 193, Japan). The supercritical fluid used was CO2 with 3% methanol added as polar modifier. Supercritical fluid extraction (SFE) was conducted under the following experimental conditions: flow rate of the supercritical fluid was 1 ml/min, the extraction cell temperature was set at 50°C, the

variable restrictor (JASCO 880-01 Back Pressure Regulator), was heated at 40°C to avoid dry ice formation, the sample was collected by bubbling in 5 ml methanol. The time of extraction was 30 minutes.

The extract was evaporated to dryness under a gentle stream of nitrogen. The dry residue was redissolved in 500 µl of methanol. This solution was filtered through a 0.22 µm membrane filter and ready for injection. Dodecanol (1 mg/ml) is added to the methanolic extract before capillary SFC analysis.

3. RESULTS AND DISCUSSION

3.1 Capillary SFC

Capillary supercritical fluid chromatography combines the high efficiency of capillary columns with the broad range of selective and sensitive GC detectors such as FID, NPD, ECD, ... Furthermore, because the working temperatures are low and the solvating power of the fluid is high in comparison with GC, it is possible to analyze thermolabile and high molecular weight compounds. Therefore, we developed a capillary SFC method which allow the simultaneous analysis of artemisinin and artemisinic acid in Artemisia Annua L. extract without a prederivatization and without degradation of the compounds. The flame ionisation detector was coupled to the SFC columns so as to obtain a good sensitivity and a large working dynamic range. Three capillary columns with increasing polarity were tested for this separation (DB-1, DB-1701, DB-WAX). As shown in figure 1, in optimized conditions, the DB-WAX column permits to separate standard solutions of artemisinin and artemisinic acid with a good resolution in less than 25 minutes. The two other columns don't give symetric peaks for artemisinic acid even if the resolution of the compounds was acceptable. In all cases, pure CO_2 was used as a mobile phase. As expected, artemisinic acid was eluted before artemisinin on DB-1 and DB-1701 capillary columns. In the case of the most polar DB-WAX column, the order of elution was inverted.

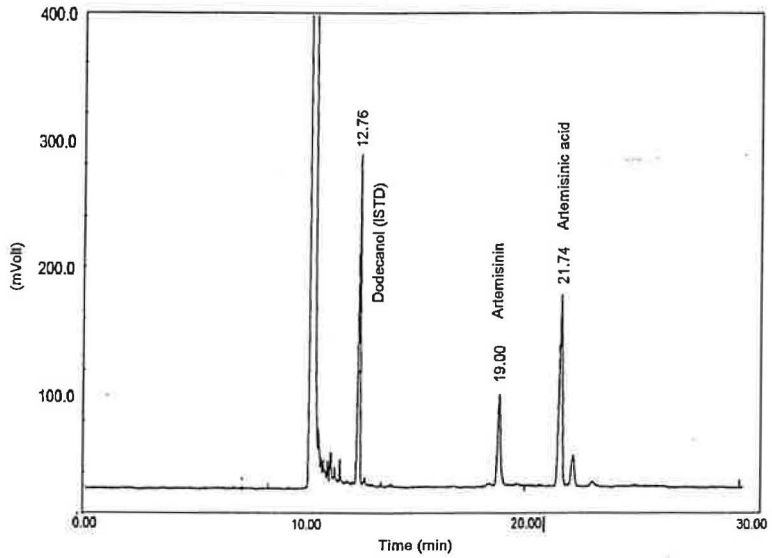

Figure 1:

SFC-FID chromatogram of artemisinin and artemisinic acid on a DB-WAX column (standard solution)

The three methods were validated and results are shown in Table 1. On the expected concentration range, the three columns gave comparable performances (i.e. repeatability, linearity, ...). We have to note that the limits of detection and quantification were better for the DB-WAX column because of its higher efficiency.

Table 1:

Validation parameters obtained on DB-1, DB-1701 and DB-wax columns.

DB-1	Artemisinin	Artemisinic acid
Repeatability (C.V. %)	4.5	7.0
Tested linearity range [mg/ml]	0.1 to 1.5	0.1 to 1.5
Coefficient of correlation	0.731, -0.028	0.919, -0.059
Slope, Intercept	0.998	0.985
Standard deviation of the slope	0.015	0.051
Standard deviation of the intercept	0.012	0.042
Limits of detection [mg/ml]	0.06	0.06
Limits of quantification [mg/ml]	0.20	0.20

DB-1701	Artemisinin	Artemisinic acid
Repeatibility (C.V. %)	2.2	2.7
Tested linearity range [mg/ml]	0.1 to 2.0	0.1 to 2.0
Coefficient of correlation	0.996	0.997
Slope, Intercept	0.760, -0.042	0.898, -0.058
Standard deviation of the slope	0.020	0.019
Standard deviation of the intercept	0.021	0.021
Limits of detection [mg/ml]	0.04	0.04
Limits of quantification [mg/ml]	0.125	0.140

DB-WAX	Artemisinin	Artemisinic acid
Repeatibility (C.V. %)	5.7	4.3
Tested linearity range [mg/ml]	0.1 to 2.0	0.1 to 2.0
Coefficient of correlation	0.999	0.999
Slope, Intercept	0.604, -0.017	1.008, -0.045
Standard deviation of the slope	0.0087	0.009
Standard deviation of the intercept	0.010	0.011
Limits of detection [mg/ml]	0.03	0.02
Limits of quantification [mg/ml]	0.1	0.07

For plant extracts analysis, artemisinin can be determined with all three columns but only DB-WAX allows to separate artemisinic acid from interfering compounds present in the extracts, as shown in figure 2.

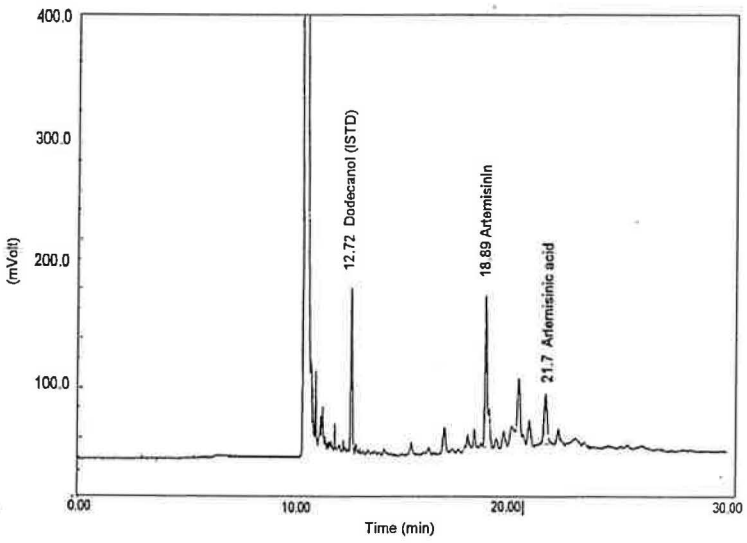

Figure 2 :

SFC-FID chromatogram of an Artemisia annua L. extract obtained by SFE.

Quantitative results obtained by SFC-FID on DB-WAX column for five plant extracts are reported in Table 2. These results were confirmed by an adapted GC-MS procedure [10] and gave comparable results. However, in the case of artemisinin analysis by GC-MS, only degradation products can be determined because artemisinin undergoes thermal degradation above 160°C.

Table 2:

Comparison between SFC-FID and GC-MS on Artemsisia annua L. extracts obtained by SFE. Concentrations are given in % dry weight.

Sample	Capillary SFC-FID		GC-MS	
	Artemisinin	Artemisinic acid	Artemisinin	Artemisinic acid
1	0.73	0.19	0.70	0.23
2	0.13	1.43	0.08	2.00
3	0.96	0.07	0.90	0.03
4	0.31	0.13	0.33	0.18
5	0.69	0.64	0.59	0.77

3.2 Packed SFC

In order to obtain faster separation and better injection repeatabilities than with capillary SFC-FID, we developed a packed SFC method coupled with an evaporative light scattering detector (packed SFC-ELSD). In order to interface the column with the detector a pinched restrictor was directly introduced into the nebulisation chamber of the ELSD.

As previously, we tested two stationary phases: a polar bare silica material and an hydrophobic C18 silica material. With both columns the compounds were retained by an adsorption mechanism, therefore a small amount of a polar modifier was necessary. In all cases, the amount of the polar modifier did not exceed 3% (v/v).

In optimized conditions, the two columns allow to separate artemisinin and artemisinic acid in less than 3 minutes as shown in figure 3.

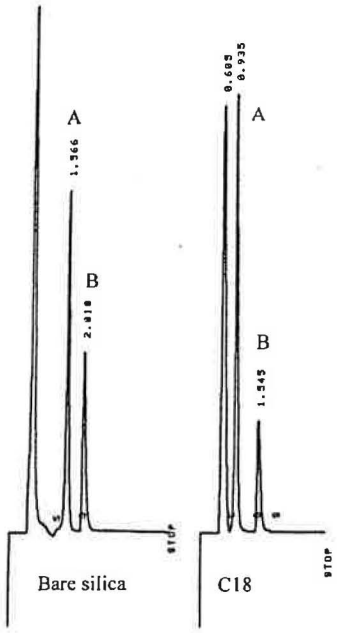

Figure 3:

SFC-ELSD chromatogram of artemisinin (A) and artemisinic acid (B) (standard solution) on a bare silica column (1.6% methanol added as polar modifier) and on a C18 column. (0.8% methanol added as polar modifier). In both cases the flow rate was set at 4.0 ml/min and the pressure was 250 bar.

The order of elution of artemisinin and artemisinic acid is the same on both columns, because the mechanism involved in the separation is the adsorption. However, the capacity factors obtained on bare silica were greater than those obtained on C18 silica because there are more available adsorption sites. Therefore, due to the complexity of the plant extract, only the bare silica was chosen for the separation of artemisinin and artemisinic acid to obtain a sufficient selectivity. Presently, in the conditions shown in figure 3, the method presents comparable results with those obtained by capillary SFC-FID. Works are in progress in order to find the best chromatographic conditions for the quantitative determination of the two compounds in plant extracts.

4. CONCLUSION

Two supercritical fluid chromatography methods have been developed in order to determine directly artemisinin and artemsinic acid. The analysis of these compounds were carried out in less than 25 minutes on a capillary DB-WAX column with pure CO2 and in less than 3 minutes on a packed bare silica column with a mixture of CO2 - methanol (98.4 : 1.6 % v/v). The optimized method allows the determination of artemisinin and artemisinic acid with a FID and an ELSD, coupled respectively

with capillary and packed column. Finally the capillary SFC-FID analytical method has been validated in the concentration range of interest and results obtained on plant extracts were verified by GC-MS. SFE allows to obtain clean plant extracts for analysis.

5. REFERENCES

1 D. L. Klayman, Qinghaosu (Artemisinin): An Antimalarial Drug from China, Science, vol 228 (1985), pp1049-1055

2 H.J. Woerdenbag, C.B. Lugt, N. Pras, Artemisia annua L.: a source of novel antimalarial drugs, Pharmaceutisch Weekblad Scientific edition, vol 12(5) (1990), pp 169-181

3 T.T. Hien, N.L. White, Qinghaosu, The Lancet, vol 341 (1993), pp 603-608

4 T. Ravindranathan, M..A. Kumar, R.B. Menon, S.V. Hiremath, Stereoselective synthesis of artemisinin, Tetrahedron Letters, vol 31(5) (1990), pp 755-758

5 Zhao Shisan, Zeng Mei-Yi, Application of precolumn reaction to high performance liquid chromatography of ginghaosu in animal plasma, Analytical chemistry, vol 58 (1986), pp 289-292.

6 N. Acton, D.L. Klayman, I.J. Rollman, Reductive electrochemical HPLC assay for artemisinin (Qinghaosu), Planta Medica, (1985), pp 445-446.

7 N. Pras, J.F. Visser, S. Batterman, H.J. Woerdenbarg, T.M. Malingré, C.B. Lugt, Laboratory selection ot artemisia annua L. for high yielding types, Phytochemical analysis, vol 2 (1991), pp 80-83

8 H.J. Woerdenbag, N. Pras, N. Gia Chan, B.T Bang, Artemisinin, related sesquiterpenes, and essential oil in Artemisia annua during a vegetation period in Vietnam, Planta med., vol 60 (1994), pp 272-275

9 M.Jaziri, B. Diallo, M. Vanhaelen, J. Homos, K. Yoshimatsu, K. Shimomura, Immunodetection of artemisinin in Aretemisia annua cultivated in hydrophonic conditions, Phytochemistry, vol 33(4) (1993), pp 821-826

10 H.J. Woerdenbag, N. Pras, R. Bos, J.F. Visser, H. Hendriks, T.M. Mallingré, Analysis of Artemisinin and related sesquiterpenoids from Artemisia annua L. by combined Gas Chromatography/Mass Spectrometry, Phytochemical analysis, vol 2 (1991), pp 215-219