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Investigation of porous graphitic carbon at high-temperature liquid chromatography with evaporative light scattering detection for the analysis of the drug combination artesunate—Azithromycin for the treatment of severe malaria

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ABSTRACT

Artesunate combined therapies represent the best option for the treatment of malaria and require the development of new methods of analysis. Retention, selectivity and detection with high-temperature liquid chromatography-porous graphitic carbon-evaporative light scattering detection was studied for artesunate and azithromycin separation. Organic solvent, concentration of organic modifiers, temperature and flow rate were all relevant parameters to optimize this separation. The behaviour of artesunate in the tested conditions appeared close to a neutral compound. In CH_3OH, only azithromycin retention was dramatically altered depending on the [triethylamine]/[formic acid] ratio and on the temperature, whereas in CH_3CN, azithromycin, artesunate, artemisinin and dihydroartemisinin retentions decreased with the temperature increase whatever the organic modifier ratio. The best efficiency was obtained with CH_3CN. 25% variation of the concentration values of the organic modifiers did not significantly influenced the retention. The sensitivity of ELSD increased with the flow rate decrease. Peak area and S/N ratio dramatically decreased with the flow rate increase by 10- and 5-fold for artesunate and azithromycin, respectively. Non-linear calibration curves were obtained for both artesunate and azithromycin.

1. Introduction

Combination therapies currently represent the treatment of choice to delay the emergence of resistance from various infectious diseases [1]. The emergence of multi-drug resistant strains of *Plasmodium falciparum*, the infectious agent responsible for malaria, is a major public health threat in many developing countries. Since 2001, the World Health Organisation (WHO) recommends the use of artemisinin combined treatments including an artemisinin derivative and another antimalarial drug.

Artesunate (AS) is the most widely available and widely used among artemisinin derivatives for the treatment of malaria [2,3]. AS is synthesized from artemisinin, extracted from the Asian plant *Artemisia annua*. AS in aqueous solutions is rapidly hydrolysed to its active metabolite dihydroartemisinin (DHA). Therefore DHA is both an *in vivo* metabolite and an artemisinin derivative therapeutically active against malaria, and AS acts as a pro-drug [4]. Tetracycline derivatives have proven to be very effective for combination treatment of malaria [5]. Azithromycin (AZ), an antibiotic with activity similar to that of tetracyclines against malaria in vitro [6,7] and *in vivo* [8], has clear advantages for malaria-related indications. AZ in combination with artemisinin derivatives exerts additive to synergistic interactions, shows no cross-sensitivity with traditional antimalarials, and has substantial antimalarial activity on its own [9].

In addition, AZ present a great activity against many bacterial agents responsible for upper respiratory infections, disease occurring in children and very often mistreated as malaria and causing death in malaria endemic areas. Therefore, the combination AS–AZ is currently under development for paediatric use as an emergency treatment of sepsis in children leaving in malaria endemic areas. The aim of this work is to provide a chromatographic system for

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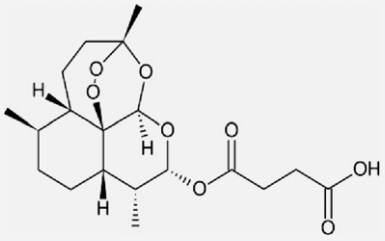
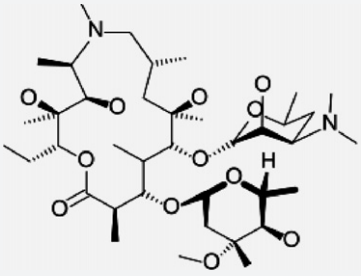
Table 1
Chemical features of AS and AZ (physicochemical data from literature).

	Chemical structure	pK_a	MW	Log *P*	Solubility
AS		4.6	384	3.5	Lightly soluble in water Very soluble in dichloromethane Freely soluble in ethanol and acetone
AZ		8.79.5	749	4.0	Practically insoluble in water Freely soluble in anhydrous ethanol and in dichloromethane

quality control of both AS and AZ in combination. To reach this goal, several elements have to be taken into account. These molecules are both hydrophobic, poorly soluble in water, without a suitable chromophore group exploitable in UV which makes their specific and sensitive detection by UV detection difficult (Table 1). In addition, AS and AZ possess acidic and basic properties, respectively. Consequently quantitative determination of AS in RP-HPLC was performed with grafted silica column using hydro-organic mobile phase with acidic buffer and detection by standard spectrophotometric detection [10–12], electrochemical detection [13–16], MS [16–20] and more recently evaporative light scattering detection (ELSD) [21,22]. The pH of aqueous part of the mobile phase was principally set at pH between 3.0 and 5.5. Whereas quantitative determination of AZ performed in RP-HPLC was with detection by standard spectrophotometric detection [23–27], and more recently by MS [28–31] using hydro-organic mobile phase with closely neutral or basic pH. Although ELSD is not to our knowledge used to detect AZ, other macrolides were successfully detected by this detector [32,33]. AZ detection with ELSD may be also implemented. In regard to the literature in RP-HPLC with grafted silica, the eluotropic strength and the pH of the mobile phase appeared poorly compatible for the simultaneous analysis of AZ and AS. Other retention mechanism may be envisaged using porous graphitic carbon (PGC) which is an LC stationary phase developed by Knox and Ross an alternative to the commonly used RP-silica packings [34]. The overall retention on a PGC column involves two major mechanisms: (1) dispersive interaction between analyte–mobile phase and analyte–graphite surface and (2) dipolar and ionic interaction of a polar analyte with the polarizable graphite surface [35,36]. In addition, PGC stationary phase is composed of almost 100% carbon with no bonded chemistry, which provides material very stable over the entire pH range and at high-temperature up to 200 °C [37].

The goal of this experimental was to assess the combination of high-temperature LC-PGC-ELSD for separation of AZ and AS and to establish the parameters controlling their retentions. These chromatographic systems may provide some key for quality control, stability study or pharmacokinetic perspectives.

2. Experimental

2.1. Chemicals and reagents

AS was a generous gift of Drug for Neglected Disease initiative (DNDi) (Geneva, Switzerland) purchased from Knoll BASF Pharma (Liestal, Switzerland). β-Dihydroartemisinin was synthesized from COMIPSO (Bordeaux, France). AZ was purchased from Pfizer (USA). Acetonitrile, methanol, dichloromethane, isopropanol, formic acid (FA) and triethylamine (TEA) were isocratic HPLC grade purchased from Prolabo VWR (Leuden, Belgium). Absolute ethanol was purchased from Cooper (Melun, France). Artemisinin was purchased from Sigma (Saint Quentin Fallavier, France).

2.2. Instrumentation and chromatographic conditions

The HPLC system was a Hewlett Packard HPLC 1050 series which consisted of a quaternary pump, an online degasser, an Agilent Chemstation LC 3D and Rheodyne 7125 injection valve with a 5 μl sample loop (Cotati, California, USA). The ELSD was CHROMACHEM (Eurosep, France) with air as nebulization gas. Column was hypercarb (50 mm × 3 mm I.D., 5 μm particle size) (Thermo Fisher, France), thermostated with Crococil oven (CIL, Saint Foy la Grande, France). A 50 cm capillary tube with 0.1 mm I.D. in the oven allowed the preheating of the incoming mobile phase.

3. Results and discussion

The separation of AS and AZ in a unique run was firstly attempted with octadecyl silica stationary phase and UV detection. However according to their pK_a and log *P* values (Table 1) there is no pH condition leading to the ionization suppression of the both compounds together. The pH value at 5.2 of the mobile phase aqueous part affords the elution of both molecules in the same isocratic method. Unfortunately both compounds are ionized. However poor detectability of AZ and poor method robustness were obtained that led us to envisage another chromatographic system constituted by PGC and ELSD.

Table 2
Solvent selection for AS and AZ using Hypercarb stationary phase.

	Isocratic elution		Gradient elution[a]			
	CH_3OH[b]	CH_3CN[b]	CH_3OH[b] Dichloromethane[c]	CH_3OH[b] Propanol-2[c]	CH_3CN[b] Dichloromethane[c]	CH_3CN[b] Propanol-2[c]
AZ						
t_R (min)	21.5	23.5	8.7	9.6	10.5	11.8
N	1620	1918	–	–		
AS						
t_R (min)	29.7	9.2	10.0	12.3	6.5	7.4
N	1815	3599	–			

[a] Gradient elution 100% weak solvent to 100% strong solvent in 30 min at room temperature, injection volume 5 μl, flow rate 0.5 ml min^{-1}. [AZ] and [AS] = 0.2 g l^{-1}. All tested solvents contained [TEA] = 36 mM and [FA] = 26 mM.
[b] Weak solvent.
[c] Strong solvent.

3.1. Solvent selection with PGC

PGC commonly employs water, methanol and acetonitrile as the mobile phase for the elution of polar compounds but requires much stronger organic solvents such as dichloromethane and tetrahydrofuran for the elution of non-polar analytes. As both molecules were poorly soluble in water (Table 1), acetonitrile (CH_3CN) and methanol (CH_3OH) were first tested as mobile phase. Without any acido-basic modifier in the mobile phase, no elution of AZ occurred and a tailing peak was obtained for AS. TEA and FA were added in the mobile phase to fix the ionization state of these molecules and were volatile enough for ELSD-compatible pH control [38]. With CH_3CN and CH_3OH, elution was obtained and the order of retention was different (Table 2). However in both cases, the efficiency was poorer for AZ than AS and the retention time important for the both molecules. In order to test other solvents, binary gradients were performed with CH_3CN or CH_3OH as weak solvent. Dichloromethane provide stronger eluotropic strength and better efficiency than propanol-2, independently of the weak solvent.

Then the influence of TEA and FA content was assessed in concentration ratio of [TEA]/[FA] between 0.5 and 2.0 in several mobile phases composed of CH_3CN or CH_3OH at 333 K. These ratios were performed using each organic modifier between 15 and 45 mM. Below 15 mM degradation of peak shape sharply occurred and over 45 mM especially with TEA background noise with ELSD increased. Whereas with CH_3CN retention of AS and AZ appeared independent of the TEA and FA concentrations on the investigated range, with CH_3OH only the retention of AZ increased with the increase of [TEA]/[FA] ratios (Fig. 1). Eluotropic strength of CH_3CN appeared higher than CH_3OH for AS. Better efficiency was obtained using CH_3CN, especially for AZ. To demonstrate that the presence of these organic modifiers influenced the retention by modifying the state of ionization of the compound instead of the modification of the PGC surface, artemisinin as neutral products was tested in those conditions and also without any modifier. The retention of artemisinin was found independent of TEA and FA contents: retention times of 4.9 and 2.3 min were found in CH_3OH and CH_3CN, respectively. Therefore the contribution on the retention of such modifiers was mainly due to modification of state of ionization of the analyte. In CH_3OH, the increase of AZ retention was correlated with the increase of the [TEA]/[FA] ratio in the mobile phase. The use of higher or smaller concentrations of these organic modifiers leading to the same [TEA]/[FA] ratio did not significantly modify the retention. pK_a values of acids increase upon the addition of organic solvent, whereas the pK_a values change to more acidic values for basic compounds. This means that the addition of organic solvent results in a weakening of both acids and bases [39,40]. ${}^{s}_{w}$pH values were measured to assess the ionization state of AZ and AS in the various mobile phase compositions (Table 3). As the retention of

Table 3
${}^{s}_{w}$pH of mobile phase.

Mobile phase composition	[TEA] (mM)	[FA] (mM)	${}^{s}_{w}$pH
CH_3CN	26.5	26.5	8.09
CH_3CN	20	26.5	7.79
CH_3CN	26.5	20	8.69
CH_3CN:CH_3OH 80:20	26.5	26.5	8.02
CH_3CN:CH_3OH 80:20	20	26.5	7.22
CH_3CN:CH_3OH 80:20	26.5	20	8.57
CH_3CN:CH_3OH 50:50	26.5	26.5	7.58
CH_3CN:CH_3OH 50:50	20	26.5	6.63
CH_3CN:CH_3OH 50:50	26.5	20	8.53
CH_3OH	26.5	26.5	7.06
CH_3OH	20	26.5	6.04
CH_3OH	26.5	20	8.08

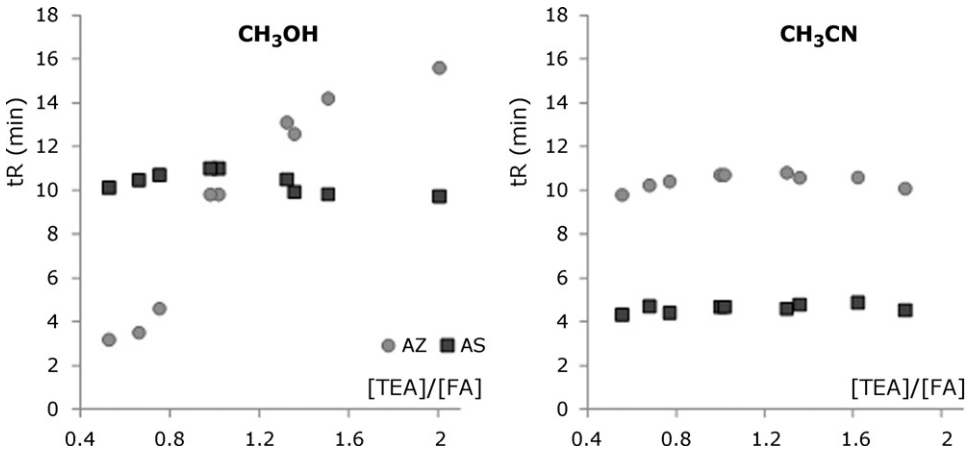


Fig. 1. Retention of AZ and AS in CH_3OH or CH_3CN versus concentration ratios of [TEA]/[FA] in the mobile phase at 0.5 ml min^{-1} and 333 K.

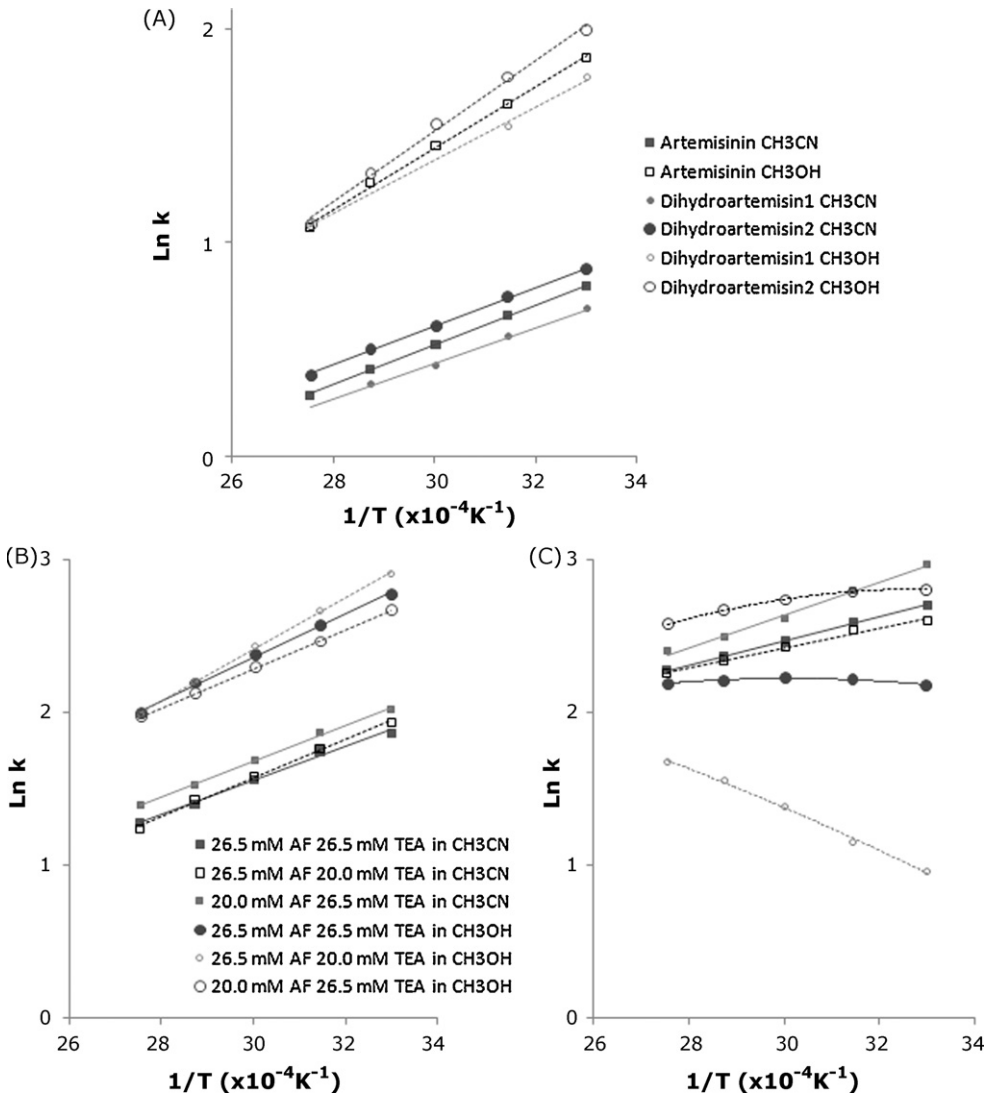


Fig. 2. Van't Hoff plots for dihydroartemisinin and artemisinin (A), AS (B) and AZ (C). Mobile phase at 1 ml min^{-1}. Concentrations of standards: AS at 0.16 mg ml^{-1}; AZ at 0.19 mg ml^{-1}.

AS remains unchanged with the tested [TEA]/[FA] ratio whatever the organic solvent used for the mobile phase that means the ionization state of AS remains the same in all tested conditions. Even if the pK_a value of AS should increase in organic solvent compared into water, the mobile phases were composed of FA and TEA which ensured to keep AS at its ionized stage. Apparently the same observation can be performed in CH_3CN for AZ which seemed stayed in its molecular state whatever the tested [TEA]/[FA] ratio. However in CH_3OH, the range of tested [TEA]/[FA] ratio led to a larger range of $^{s}_{w}$pH than in CH_3CN which induced modification of the ionization state of AZ. Therefore this range included the pK_a value of AZ in CH_3OH.

Although using CH_3CN as mobile phase had the advantage of better efficiency (approximately twice than with CH_3OH mobile phase), this solvent prevented the modulation of the selectivity AS and AZ in the range of tested [TEA]/[FA] ratios.

3.2. Temperature study on the retention

The separation of ionizable compound mixtures involved the optimization of mobile phase composition and also the temperature because temperature plays a key role on dissociation constants of ionizable solutes which affects the retention and thus the selectivity [41]. As the ionization state of AZ was modified depending on the solvent and organic modifiers in the mobile phase, the influence of the temperature on the retention was studied by Van't Hoff plots ($\ln k$ as a function of $1/T$).

Van't Hoff plots were established with mobile phases composed by CH_3OH or CH_3CN testing at three different [TEA]/[FA] ratios for neutral, acidic and basic compounds (Fig. 2). The range of investigated temperatures was between 303 and 363 K. These plots were fitted with a linear model with correlation coefficients $R^2 > 0.997$ and 0.992 for neutral and ionizable compounds, respectively. Except for data obtained with AZ in CH_3OH conditions, a quadratic model was applied ($R^2 > 0.970$). Linear Van't Hoff plots are usually reported for neutral compounds with temperatures up to 90 °C [42], suggesting that ΔH° and ΔS° are essentially temperature-independent over the entire range of examined temperatures, whatever the mobile phase composition. In addition, the same plots were obtained for the neutral compounds without organic modifiers (i.e. TEA and FA) that confirmed the retention mechanism is not due to an association of the organic modifiers with PGC leading to the surface modification [43]. However in the range of studied temperatures AS led also to a linear behaviour, consequently this nonclassical curvilinear Van't Hoff relationship for AZ was rather attributed to a change of heat capacity of the sys-

tem AZ-mobile phase dependent on temperature instead of PGC surface modification due to temperature or organic modifier addition. This phenomenon is likely to be consistent with a change in the retention mechanism due to the change of ionization state of AZ with the temperature in CH_3OH for the range of tested combinations of [TEA/[FA]. In CH_3OH, by selecting the [TEA/[FA] ratio AZ retention can increase ([TEA/[FA] < 1), decrease ([TEA/[FA] > 1) or remain ([TEA/[FA] = 1) stable with temperature increase.

The retention of AS decreased with the temperature in all tested conditions and the Van't Hoff plots fitted with linear relationships. The retention of AS was more important with CH_3OH than CH_3CN whatever the modifiers in the mobile phase. In addition, the retention of AS was always higher than artemisinin and dihydroartemisinin independently of the mobile phases.

As the Van't Hoff plot of AS in the tested conditions appeared linear and also for AZ only in CH_3CN mobile phases, we can conclude that the ionization state remained unchanged. However in the range of tested temperature and organic modifiers only the ionization state of AZ appeared modify and only in CH_3OH. Ionizable compounds led to more complex behaviour of Van't Hoff plots, since solute may be under both neutral and ionized forms which led to different retention enthalpy and the entropy. There is no significant shift of the pK_a value for weak acid in relation with the temperature [44]. The temperature was an important parameter to assess for mobile phase optimization, especially for basic compounds for which a balance between a retention decrease due to lower hydrophobic interaction in the neutral state of solute and a retention increase due to a decrease of dissociation rate may occurs, that explained the non linear Van't Hoff curves of AZ.

3.3. Separation optimization

The temperature, solvent and organic modifiers of the mobile phase were all parameters relevant to adjust the separation between AS and AZ:

(i) Whereas in CH_3OH, retention of AZ was dramatically altered depending on the [TEA]/[FA] ratio and the temperature instead of AS retention, artemisinin and dihydroartemisinin retentions which always decreased with temperature increased whatever the modifier ratio, in CH_3CN.
(ii) The solvent selection influenced AZ efficiency. The best efficiency was obtained with CH_3CN.
(iii) The organic modifiers expressed as [TEA]/[FA] ratios modified the retention of AZ in CH_3OH only. And 25% variation of the concentration values of the organic modifiers did not influenced significantly the retention.

Therefore in CH_3CN, a unique separation between AS and AZ can be obtained, increasing the temperature was useful to strictly decrease the analysis run time. For this reason the partial use of CH_3OH in the mobile phase was considered even if this solvent decreased efficiency. Binary isocratic mobile phases were investigated with TEA and FA at 20 and 26 mM of TEA and FA concentrations, respectively, because reversal of elution order was achieved for AS and AZ depending on the solvent nature between CH_3OH and CH_3CN. In addition to this modifier content, the retention of AZ increased with the temperature increase. Adding CH_3OH in the mobile phase constituted by CH_3CN rapidly decreased the retention of AZ, whereas the retention of AS slightly increased (Fig. 3). As with 20% of CH_3OH in CH_3CN, the efficiency remains identical, this CH_3OH content was selected and temperature increase was tested. Increasing temperature improved resolution between AS and AZ as expected (Fig. 4). Although increasing the temperature in that condition increased the run time, there was an advantage with the highest temperature that dihydroartemisinin

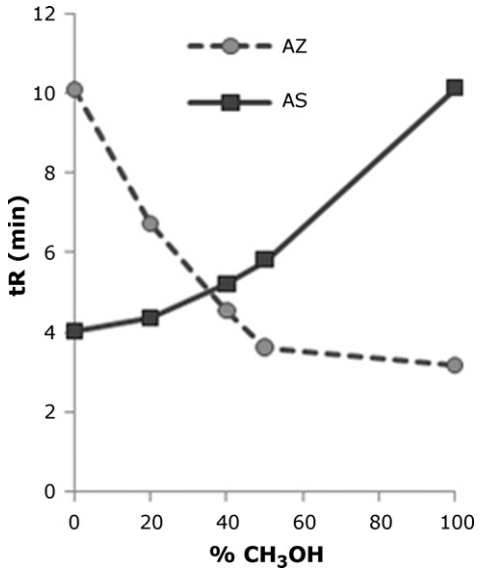


Fig. 3. Retention of AZ and AS in binary mobile phases of CH_3OH and CH_3CN. 26.5 mM FA 20 mM TEA. Mobile phase at 0.5 ml min^{-1} and 333 K.

was analysed as a unique peak. β-Dihydroartemisinin in solution led to α and β epimers [45] which were separated with HPLC [46]. Consequently the quantification of this AS degradation product can be improved and its LOD decreased.

As the content of organic modifiers in combination with the presence of CH_3OH in the mobile phase influenced the retention, chromatographic parameters were compared at various [TEA]/[AF] ratios from 0.75 to 1.32 which represents a variation of ±25% around the value of 1. In spite of the variation of AZ retention with [TEA]/[AF] ratio, the impact on AZ area appeared weak. In fact DHA area were the most influenced mainly due to asymmetry variation induced by the presence of two epimers. However these results were obtained with a large range of ratio variation and provided some elements of robustness of this analytical system for quantification purpose since the combination of FA and TEA is not a buffer.

3.4. Detection study

In this study the use of PGC column contributes to another advantage for this compound separation that the exclusive use of

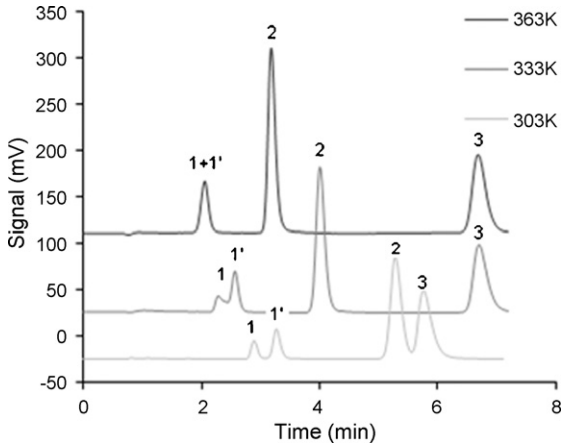


Fig. 4. Separation of dihydroartemisinin, AS and AZ with PGC stationary phase at different temperatures. Isocratic mobile phase 80:20 CH_3CN:CH_3OH + 25.6 mM FA + 20.0 mM TEA, 90 °C, 0.5 ml min^{-1}. 1 and 1′: DHA 0.05 mg ml^{-1}; 2: AS 0.01 mg ml^{-1}; 3: AZ 0.01 mg ml^{-1}.

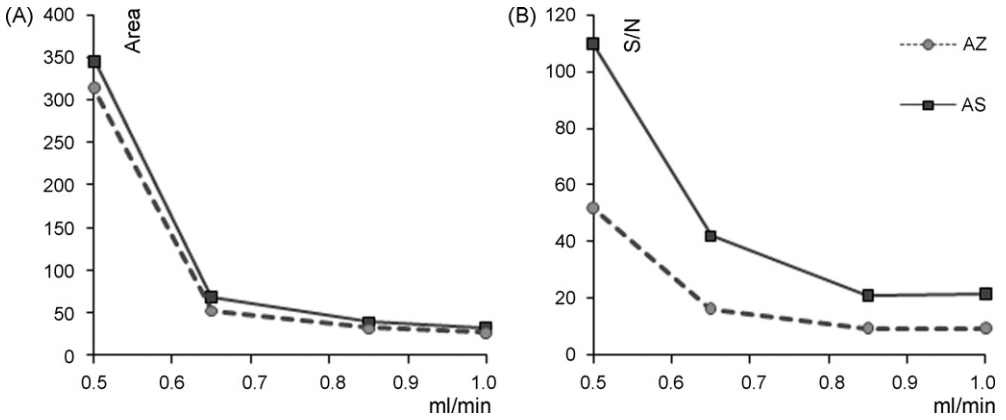


Fig. 5. ELSD signal of AS and AZ versus mobile phase flow rate. Isocratic mobile phase 80:20 CH_3CN:CH_3OH + 25.6 mM FA + 20.0 mM TEA, 90 °C. 0.16 mg ml^{-1} AS; 0.19 mg ml^{-1} AZ. ELSD parameters: nebulization temperature: 35 °C; evaporation temperature: 50 °C; air set at 1.5 bar.

organic solvents as mobile phase components was advantageous for ELSD.

A claimed advantage of high-temperature liquid chromatography (HTLC) due to viscosity decrease of the mobile phase with the temperature increase is the increase of the flow rate for decreasing the time analysis [47]. Moreover it is clearly demonstrated that increasing the flow rate in HTLC do not involve an important decrease of the efficiency [48,49]. This option was envisaged and studied with our conditions with regard to ELSD.

During the nebulization step, the droplet size is related to the surface volume mean droplet diameter, D_{sv} (Sauter mean diameter), calculated by the Nukiyama/Tanasawa empirical equation (1) [50]:

$$D_{sv} = \frac{585\sqrt{\sigma}}{\nu_g - \nu_l} + 597\left(\frac{\mu}{\sqrt{\sigma\rho}}\right)^{0.45}\left(1000\frac{Q_l}{Q_g}\right)^{1.5} \quad (1)$$

where σ is the mobile phase surface tension, ρ its density, μ its viscosity, $\nu_g - \nu_l$ the difference between the nebulizer gas and liquid velocities, Q_l/Q_g the ratio of liquid and gas volumetric flow rates. Equation (1) shows that D_{sv} depends on the Q_l/Q_g ratio and $(\nu_g - \nu_l)$ when the flow rate of the mobile phase was altered. Therefore the detection would be modified because D_{sv} would increase with the flow rate increase. The area and S/N were measured for flow rate between 0.5 and 1 ml min^{-1} (Fig. 5). Peak area and S/N ratio dramatically decreased with the flow rate increase by 10- and 5-fold for AS and AZ, respectively. The decrease of the S/N with the flow rate increase was mainly due to the signal decrease instead of a background noise increase. Therefore the decrease of the area was mainly related to the particle size decrease. Although increasing the flow rate contributed to increase the droplet size, it also contributed to the elimination of a higher number of the biggest droplets and to dilute the solutes in the mobile phase. This latter influence was meaningful for the signal decrease. Bigger droplet contained less compound which led to smaller particles after the evaporation process. With ELSD, the increase of the flow rate for decreasing the time analysis was clearly not an advantage for the signal intensity that confirms previous results [22]. Non-linear calibration curves were obtained for both AZ and AS which was usual with ELSD. The calibration curves were $Y = 107876X^{1.4072}$ with $r^2 = 0.9992$ and $Y = 83403X^{1.339}$ with $r^2 = 0.9976$, for AS and AZ, respectively. The responses appeared similar for the both compounds. The LOD were established (Table 4). These values are better than the other individual methods reported in the literature which used UV detection [22,25]. LOD of dihydroartemisinin was better in isocratic condition at elevated temperature instead of gradient at room temperature. For AZ and AS values were of the same order of magnitude.

Table 4
LOD.

	Gradient[a] (μg ml^{-1})	Isocratic[b]
DHA	30	20
AS	1.5	2
AZ	1.5	4

[a] Gradient from 80:20 CH_3CN/CH_3OH to 100% DCM with a slope at 3.3%/min, [TEA]/[FA] = 1 at room temperature and 0.5 ml min^{-1}.
[b] Isocratic 80:20 CH_3CN/CH_3OH [TEA]/[FA] = 0.75, 363 K, 0.5 ml min^{-1}.

4. Conclusion

The optimization of the separation between AS and AZ can be obtained with an noteworthy flexibility using PGC with mobile phase composition based on the selection among three parameters (i.e. solvent, [TEA]/[FA] ratio and temperature). However the important modulation of the selectivity was mainly afforded by the versatile AZ retention in CH_3OH since AS retention appeared slightly influenced by [TEA]/[FA] ratio. Increasing temperature gives the advantages of affording AZ elution with good peak shape and dihydroartemisinin retention as a unique peak at high-temperature.

The main drawback of the method was the use of mobile phase containing 100% organic solvent for cost-effective and environmental reasons. However conventional HPLC method for AZ currently used solvent content in the mobile phase up to 80% [27]. For ELSD and ESI source, this high solvent content of the mobile phase was an advantage which improves the effectiveness of nebulization and evaporation process resulting in better sensitivity of the analysis. Better sensitivity of a LC–MS method is generally measured with mobile phases containing high organic content in ESI because of lower surface tension and therefore better spraying. PGC columns use MS-friendly mobile phases, and therefore enable efficient coupling with ESI-MS. The mobile phase remained compatible with MS detection. So this method may be hyphenated with MS. In addition the analysis of artemisinin as internal standard for quantification purpose with LC/MS in the same run of AS and AZ is possible with this chromatographic system.

PGC-HTLC-ELSD system was proved to be efficient and flexible for AZ and AS separation and thus may be useful for AZ/AS formulation control and offers a great potential for stability study and pharmacokinetic study.

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