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Short communication

Detection and separation of artesunate and artelinic acid with capillary zone electrophoresis

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1. Introduction

Artesunate and artelinic acid are two semisynthetic compounds obtained after hydration and respectively ester- and etherification of artemisinin. Artemisinin is a very effective antimalarial compound of the Chinese traditional herb, *Artemisia annua* L. Their pharmacological necessity has called for intensive chemical and pharmacological studies. Artesunate lacks functions suitable for quantitative evaluation. Several techniques were developed for artesunate but most require very specialized analytical conditions.

Capillary zone electrophoresis (CZE) has several advantages over other ion analysis techniques, even over HPLC: high separation efficiency, short run time, very small sample take-up and direct sample injection with little treatment [1]. The possibilities of ionization of artesunate and artelinic acid were decisive in the selection of CZE. Despite some disadvantages such as low sensitivity and loadability [2], the CZE-technique can be useful for analysis in pharmaceutical preparations, giving high dose samples. Earlier studies [3] have been reported in this domain: development of a CZE-technique for the analysis of artesunate in the presence of its hydrolytic degradation products, dihydroartemisinin and succinic acid.

In our study, a CZE-technique for the separation of two structurally comparable products, artesunate and artelinic acid is described and the influence of several parameters was investigated. A more sophisticated variant, the micellar electrokinetic capillary electrophoresis, generating micelles after addition of surfactants, was also investigated to improve the separation efficiency and sensitivity.

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2. Experimental

2.1. Chemicals and reagents

The active compounds used in our study, artesunate and artelinic acid were purchased respectively from Helm AG (Hamburg, Germany) and ACF-Beheer (Maarssen, The Netherlands). The organic solvents, acetic acid 96% (v/v) and acetonitrile, having pro analysi quality, and methanol with a lichrosolve gradient quality, were obtained from Merck (Darmstadt, Germany). The following compounds, disodiumhydrogenopyrophosphate, purchased from Aldrich (Beerse, Belgium), sodiumhydrogenophosphate monohydrate from Merck (Darmstadt, Germany) and SDS from Flandria (Ghent, Belgium), were used for the preparation of the buffer solutions, varying in concentration, pH, and addition of surfactants. Sodium hydroxide, used for the reaction of artesunate into its sodium salt was purchased from UCB (Brussels, Belgium). All water-containing solutions were prepared with Milli-Q water, purified with a Seral Pro 90 CN-filter.

2.2. Instrumentation

Capillary electrophoresis experiments on 100 ppm stock solutions of artesunate and artelinic acid, first prepared in respectively 0.1 N NaOH and water and later in water/methanol (1:1) (v/v), were carried out on a Waters Quanta-4000 Capillary Electrophoresis system, ambient temperature controlled and equipped with a positive power supply. The fused-silica capillaries have an inner diameter of 75 μm and a total length of 60 cm. The injection was carried out hydrodynamically over 10–60 s depending on the tests. Between each run the capillary was flushed with 0.01 N potassium hydroxide and Milli-Q water, each during 1 min, to clarify the capillary. The direct method was tested, whereas the electrophoreting zones were detected with a fixed wavelength of 185 nm. The electropherograms were recorded and integrated with an Intersmat/Shimadzu IC-R3A data processor.

3. Results and discussion

The physico-chemical properties of the mobile phase, changing the characteristics of the buffer (pH, concentration and type) or adding organic modifiers or tensioactive compounds, were studied. The influence of instrumental parameters on the separation behaviour of artesunate and artelinic acid were also investigated. The migration behaviour and selectivity of artesunate and artelinic acid are taken into consideration to obtain optimized conditions for the separation and detection of artesunate and artelinic acid. Even stability enhancing solvents for artesunate were tested.

3.1. Influence of physico-chemical parameters

3.1.1. Type of buffer

Two different types of buffer, based on sodium phosphate and sodium pyrophosphate salts of the same concentration (10 mM) and pH 7 were tested under the following conditions: capillary: fused silica, length 60 cm; i.d. 75μm; voltage 15 kV; injection time 30 s. The effect of both buffers was noted in the base line which was not stable with the phosphate buffer, already reported by Jimidar [4] and increased as a function of time, possibly due to an increased noise-level current.

3.1.2. pH and concentration of the buffer

The pyrophosphate buffer (10 mM; pH 7) was selected for further experiments. The effect of its pH with a concentration of 10 mM was studied in

Table 1
Influence of pH on the migration time and separation efficiency of artesunate and artelinic acid[a]

Analyte	Migration time (min)		
	pH 7	pH 7.5	pH 8
EOF	3.129	2.859	2.608
Artesunate	4.586	3.975	3.476
Artelinic acid	4.748	4.085	3.556
R_s	0.02	0.03	0.07

[a] Conditions: fused capillary: 60 cm, i.d. 75 μm; pyrophosphate buffer (10 mM); voltage 15 kV; injection time 30 s.

Table 2
Influence of the addition of SDS to the sodium pyrophosphate buffer on the migration time and separation efficiency of artesunate and artelinic acid[a]

Analyte	Migration time (min)			
	SDS (0 mM)	SDS (10 mM)	SDS (20 mM)	SDS (40 mM)
Artesunate	4.586	4.964	7.842	6.810
Artelinic acid	4.748	6.421	13.110	19.530
R_s	0.0	6.1	79.3	462.2

[a] Conditions: fused capillary: 60 cm, i.d. 75 μm; pyrophosphate buffer (pH 7; 10 mM); voltage 15 kV; injection time 30 s.

the range 7–9. The data from Table 1 show that a decrease of the pH gives a slight increase in migration time and separation efficiency. The difference of the migration time of artelinic acid at pH 7 and 8 is more than 1 min. The difference between the migration times of both analytes is smaller when the pH is increasing. For pH 7 and 8, two clear peaks of artesunate and artelinic acid were observed, but a very small improvement of the separation efficiency was obtained with pH 8. However, it is difficult to decide which pH is the most suitable for good results. With pH 9, the separation between artesunate and artelinic acid was not observed anymore, and the base line was very unstable. Finally, we selected pH 8 for further experiments. Investigation of the buffer concentration confirmed that migration time generally increases with decreasing buffer concentration [5]. Respectively 4 and 4.5 min in a 20 mM buffer solution and 6.5 and 7.5 min in a 10 mM buffer solution were observed for artesunate and artelinic acid. A 10 mM buffer gives the highest separation efficiency but increases the analysis time with almost 3 min comparing with the 20 mM buffer. Up to now, it seems to be unnecessary to test lower and higher concentrations of the buffer. For these reasons, the 10 mM concentration of the pyrophosphate buffer was selected for the following tests. However, further investigation to obtain higher separation efficiency between artesunate and artelinic acid, without increasing the analysis time, is recommended.

3.1.3. Addition of acetonitrile in the mobile phase

Table 2 shows that the addition of acetonitrile to the buffer in all cases, again increased the migration time, without any positive effect on the separation of artelinic acid and artesunate; a rather negative effect was observed.

3.1.4. Concentration of SDS in the buffer

The influence of the concentration (ranging from 10 to 40 mM) of the anionic surfactant, applied in this work, namely SDS, in the sodium pyrophosphate (10 mM) is given in Table 3.

3.1.5. Stability enhancing solvents for artesunate

As mentioned in literature [6], artesunate is soluble in alkaline aqueous solutions, forming its salt but is very unstable. Because of the insolubility of its acid, a methanolic/aqueous solution is preferred. The ratio was kept at 1:1 (v/v) which is compromising the electrical conductivity of water, necessary for CZE and stability enhancing effects on artesunate.

It had a great implication on the sensitivity of the developed technique due to the fact that injections longer than 10 s are not improving our sensitivity and are even decreasing it.

3.2. Influence of instrumental parameters

Reducing the length of the capillary negatively influences the migration time of the technique, especially for artesunate, which is not acceptable for our purpose.

The voltage did affect separation significantly, namely the higher the voltage, the lower the migration times, so, the peaks of artesunate and artelinic acid are lesser separated. The selected voltage, 20 kV gives the following results: the migration times of our analytes artesunate and

artelinic acid are respectively 6.5 and 8.7 min, compared with the results in Table 2 which give retention times of 4.9 and 6.4 min after analysis with a voltage of 15 kV. The column efficiency for both analytes on a column of 60 cm is respectively 1878 and 1892 theoretical plates for artesunate and artelinic acid.

3.3. Optimization of the CZE-technique for quantitative analysis

Since one of the goals of this investigation was to determine the optimum conditions for the quantification of artesunate and artelinic acid, the usefulness of the developed technique in a quantitative way was tested for each compound. All tests for qualitative analysis were executed on standard 100 ppm stock solutions in methanol/water (1:1) (v/v) under the following conditions: 10 mM pyrophosphate-buffer (pH 8) with 10 mM SDS, injection time 10 s and a voltage of 20 kV. The length of the capillary was kept at 60 cm. With these qualitative results, the sensitivity of the CZE-technique for artesunate and artelinic acid could already be estimated: as observed in the qualitative experiments; the peak heights for artelinic acid were always higher than for artesunate. Thus, we obtained better sensitivity results.

3.3.1. Artesunate

Concerning the sensitivity of artesunate at $\lambda =$ 185 nm, the developed CZE-technique with direct UV-detection did not reach our expectations. Concentrations lower than 100 ppm cannot be measured. The form of the peaks was not acceptable for quantitative analysis and the signal/noise range seemed to be too low. So, the detection level of artesunate is in this range, which suggests that our technique is not very sensitive for application in e.g. pharmacological studies. Nevertheless, this method can be easily applied in the domain of pharmaceutical research especially pharmaceutical products' dosage analysis, knowing that artesunate is not analyzable without derivatization in UV or with special detection apparatus as in electrochemical methods. The following experiments are thus focused on improving the detection abilities of the CZE-techniques. Normally, a positive effect on the sensitivity can be observed by increasing the injection time; a higher sample volume can be injected. Changing the injection time, varying from 10 to 60 s did not improve the sensitivity. Besides, we need to keep the injection time at 10 s because of the presence of methanol in the mobile phase.

Other possibilities for sensitivity enhancement are sample preconcentration techniques. Such techniques can be interesting in our investigation of pharmaceutical formulations because it not only serves to concentrate but also to purify the sample. Preliminary tests in this area, executed in our laboratory, seem to be hopeful and this represents a new topic in our investigation of the antimalarials.

3.3.2. Artelinic acid

For artelinic acid, the developed CZE method with direct UV-detection at 185 nm seems to be quantitatively useful; as the peak areas of artelinic acid were always higher than for artesunate, the CZE-technique seems to be more sensitive for

Table 3
Influence of the addition of acetonitrile to the sodium pyrophosphate buffer (10 mM) on the migration time and separation efficiency of artesunate and artelinic acid[a]

Analyte	Migration time (min)			
	Acetonitrile (10%)	Acetonitrile (20%)	Acetonitrile (30%)	Acetonitrile (40%)
Artesunate	3.129	4.009	4.590	5.633
Artelinic acid	3.238	4.155	4.823	5.892
R_s	0.03	0.06	0.15	0.19

[a] Conditions: fused capillary: 60 cm, i.d. 75 μm; in pyrophosphate buffer (pH 8, 10 mM); voltage 15 kV; injection time 30 s.

Table 4
Precision data ($n = 6$ or 2) of repeated injections of artelinic acid at concentrations of 5, 15, 30, 40, 50 and 60 ppm (within day and between day)[a]

		Concentration (ppm)					
		5	15	30	40	50	60
Within day precision	Mean	5.22	15.19	29.85	38.94	50.23	60.28
	R.S.D.	6.49	0.23	3.25	1.15	1.93	1.42
Between day precision	Mean	5.63	15.20	28.89	39.08	49.94	60.99
	R.S.D.	8.81	3.07	2.46	1.68	1.91	2.46

[a] R.S.D., relative standard deviation.

quantitative determination purposes. It is essential that an acceptable degree of precision could be obtained for assay investigation. The validation aspects assessed are similar to those evaluated for HPLC-methods in general and include performance parameters such as selectivity, precision, accuracy, linearity and repeatability.

3.3.2.1. Selectivity. The migration order of artesunate before artelinic acid was confirmed by the quantitative experiments. Artemisinin, arteether and artemether, three comparable products from the same group, could not be analyzed by CZE, since they migrated with the EOF and have no ionizable functional group. The results of the first tests for an internal standard, which can be applied for the development of preconcentration techniques on artesunate, prove an efficient separation of sodium benzoate from artelinic acid and artesunate, giving retention times of respectively 8.352, 7.996 and 6.118 min and 4.111 min for the EOF.

3.3.2.2. Precision and repeatability. Six repeated injections of different concentrations of artelinic acid gave the data presented in Table 4. The CZE-technique used gives reproducible migration times even after 'within day' and 'between day' analysis. The inter-day R.S.D. values for the retention time are respectively 1.58 and 1.88%. Except for the lowest concentration, 5 ppm, the results given in Table 4, are statistically significant. The separation and the quantification of artelinic acid were successfully repeated on freshly prepared solutions and different days.

3.3.2.3. Accuracy. The accuracy was tested by the determination of six samples in the range of 5–60 ppm, six times measured. The slope of the plot of the theoretical concentration against the experimental determined concentration is 1.000 and the correlation coefficient is 0.998. A small negative intercept of -0.059 is observed. The method proved to be sufficiently accurate.

3.3.2.4. Linearity. The linearity of peak area measurement for artelinic acid was assessed over the range of 5–60 ppm and gives the equation $y = 1.00239x - 0.05546$ ($R = 0.999$) with y, the peak area and x the concentration of artelinic acid in ppm. Within day R.S.D. on slope and intercept is respectively 0.017 and 0.529; inter-day values are 0.057 and 1.369.

4. Conclusion

A CZE method for the separation of artesunate and artelinic acid, used in qualitative analyses, has been successfully developed. Some advantages were observed in comparison with the study of D'Hulst and co-workers [3]. We detected the same problem of sensitivity with artesunate, but a higher selectivity of both investigated products was reached using micellar electrokinetic capillary electrophoresis, using SDS as surfactant.

The identification of both analytes yielded excellent results in that they are well separated.

Concerning quantification of artesunate, UV-detection at 185 nm was not sensitive enough.

Concerning artelinic acid, a new suitable and useful technique, namely a CZE method with direct detection at 185 nm has been developed. The method has been validated and shows good performance with respect to selectivity, linearity, precision, repeatability and accuracy. Taking these quantitative results into consideration, the technique will be very beneficial for the analysis of artelinic acid in pharmaceutical formulations. Normal doses of artelinic acid preparations are in the range of 100 mg and lower, so many chemical–pharmaceutical tests can be performed.

It is one of the first techniques, without elaborate derivatization systems, which can be adapted for the analysis of artesunate. The first steps have been made in the area of sample preconcentration and a suitable internal standard, sodium benzoate, has been found. It looks to be a very hopeful direction for continued investigations.

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