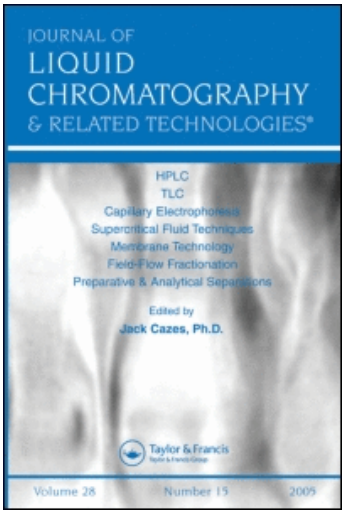

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MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY AS AN ALTERNATIVE METHOD FOR THE DETERMINATION OF SULFONAMIDES AND THEIR ASSOCIATED COMPOUNDS

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ABSTRACT

A micellar electrokinetic capillary chromatography method is presented to separate sulfamethazine, sulfaquinoxaline, menadione, and pyrimethamine. Separation was carried out at 20 kV for the five first minutes and then rised to 30 kV until the end came, using 30 mM borate buffer adjusted to pH 9.2, 6% acetonitrile, and 40 mM sodium dodecyl sulfate as electrolyte.

The limits of quantification were about 1 mg/L for every component. The method was applied in veterinary products and the results showed that some commercial claimed levels are not in agreement with the obtained results by using our analytical method, as are in other cases.

Finally, a HPLC method is used to determine the same mixture with similar results, than that provided by micellar electrokinetic capillary chromatography.

INTRODUCTION

Sulfonamides are anti-bacterial, anti-infective agents used widely in medicine and in the normal veterinary use. They are rapidly absorbed, so that therapeutic ranges of 30-150 mg/L in plasma and 500-1000 mg/L in urine[1] have been established. Using them in veterinary practice may have a risk: residues can contaminate food products coming from the animals. In many countries, maximum residue levels for sulfonamides below 1 mg/Kg have been established.

Nowadays, pharmaceutical and veterinary products frequently contain sulfonamides in conjunction with other compounds in order to increase their activities (potentiators). The therapeutic action of these compounds on the animals can often be completed with a vitamin support.

Spectrophotometric methods on the basis of the Bratton-Marshall reaction are commonly used for determining the total sulfonamide content,[2] but when the aim is the identification and quantification of individual compounds in mixtures containing sulfonamides, potenciators, and/or vitamins, the separation techniques provide analyitical methods for resolving these combinations properly.

The capillary electrophoresis (CE) techniques, including micellar electrokinetic capillary chromatography (MECC) are efficient and quick. Their development has been growing up more and more every day, specially in the biological and pharmaceutical fields.

Some references were found for the separation of vitamins[3-5] or sulfonamides[6-7] by CE. In these papers the separation of a lot of compounds belonging to the same family is carried out in order to show the high efficacy of this technique, but these methods have very limited applications in the pharmaceutical field.[8]

Since MECC has proved to be a valuable method in the quality control of drug substances,[9-11] its performance in the determination of sulfonamides, sulfamethazine (SMT), and/or sulfaquinoxaline (SQX), in association with other compounds, such as pyrimethamine (PMT) and/or menadione or Vitamin K_3 (MEN) was evaluated in this study.

This paper does research into the possibilities offered by MECC for the routine analysis of these drugs in veterinary formulations. The results were compared with those obtained by the HPLC-UV method, suggested by the authors.

EXPERIMENTAL

Material, Reagents and Solutions

Di-sodium tetra-borate anhydrous and sodium hydroxide were from Panreac (Barcelona, Spain); sodium dodecyl sulfate (SDS), sulfamethazine, sulfaquinoxaline, menadione and pyrimethamine (Figure 1) were from Sigma Chemical Co. (Germany and Switzerland).

Milli-Q water was used throughout the study.

To optimize separation, a preliminary study was carried out using a solution comtaining all components: 40, 30, 20, and 20 mg/L of SQX, SMT, MEN, and PMT respectively. Different electrloyte compositions were used to study the influence of several parameters on the separation. Three sets of electrolyte compositions were investigated:

20 mM borate buffer (pH 9.2), 30 mM SDS and variable amounts of EtOH, MeOH and MeCN;

20 mM borate buffer (pH 9.2), MeCN 6% with SDS concentrations from 0 to 80 mM;

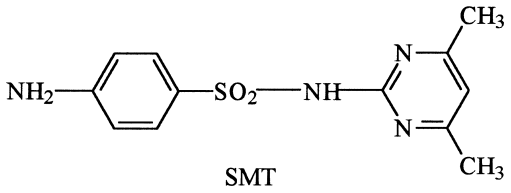
MeCN 6%, SDS 40 mM and concentrations of borate buffer (pH 9.2) between 10 and 40 mM.

The optimized electrolyte composition chosen for separation was 30 mM borate buffer (pH 9.2), 40 mM SDS, and 6% MeCN. The electrolyte solutions were employed just once for each separation batch to keep the same chemical conditions along the same sequence.

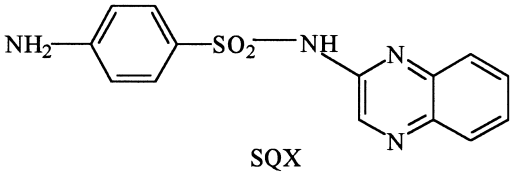
Instrumentation and Operation Conditions

A Beckman P/ACE 5500 capillary electrophoresis system equipped with a diode array detector was used. The system was controlled by a Dell Optiplex 466/L PC with Gold and Array view software.

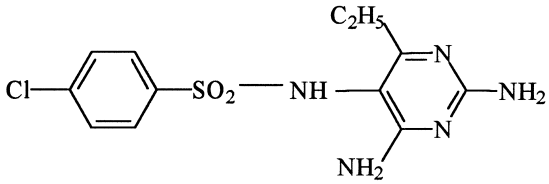
Separation was carried out in a fused silica capillary (Beckman) that was 57 cm total length (50 cm effective length), 75 μm I. D. and 375 μm O. D. and housed in a cartridge with a 800 x 100 μm detector window.



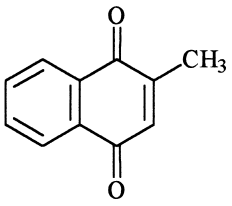
SMT



SQX



PMT



MEN

Figure 1. Chemical structure of the mixture components.

The capillary was conditioned by flushing first with 0.1 M NaOH for 30 min and then with water for 10 min before it was used for the first time. In preliminary experiments, the method used was as follows: rinsing for 3 min (high pressure) with electrolyte; then 5 s hydrodynamic injection of the mixture

standard solution. The electrolyte, operating potential, and temperature were varied according to the experiments. In the optimized method, we begin rinsing NaOH 0.1 M for 2 minutes and then with electrolyte for 3 minutes. Then afterwards hydrodinamic injection of the sample for 5 s and separation at 20 kV for 5 min with a voltage ramp of 50 kV/min. At 5 min, a second voltage ramp (until 30 kV in 0.40 min) is applied and, finally, separation is performed for 11 min.

Separate vials of electolytes were used for rinsing and separating operations in order to keep the electrolyte level in the anodic side unchanged. The set of separation vials was changed after each batch run (maximum 10 separations). Injections of the solutions among the standards were performed and peak corrected areas were used for quantification.

RESULTS AND DISCUSSION

Preliminary Investigations

SDS was selected as micellar additive in the electrolyte as it is the most common surfactant used in MECC. A borate buffer at pH 9.2 was chosen in our study due to the following reasons: first, the component pK_a values are all lower than 7.5, so at pH 9.2 it is certain that the four components are just in one form; second, the high buffer ability of the borate ($pK_{a1} = 9.2$); and third, borate buffer reports low conductivity.

Influence of the Organic Modifier

Preliminary experiences suggested addition of some kind of organic modifiers because some peaks were not well resolved and showed shoulders. The experiments were performed using 20 mM borate buffer, pH 9.2 containing 30 mM SDS as electrolyte, and with samples containing 4 mM borate buffer. The separation potential was 20 kV and the operating temperature, 25ºC. Methanol, ethanol and acetonitrile were tested in concentrations from 3 to 15 %.

Neither methanol nor ethanol got good resolution between SQX and SMT, whose peaks kept on showing their respective shoulders. However, the presence of a 6 % of acetonitrile in the electrolyte showed well resolved peaks and shoulders disappeared.

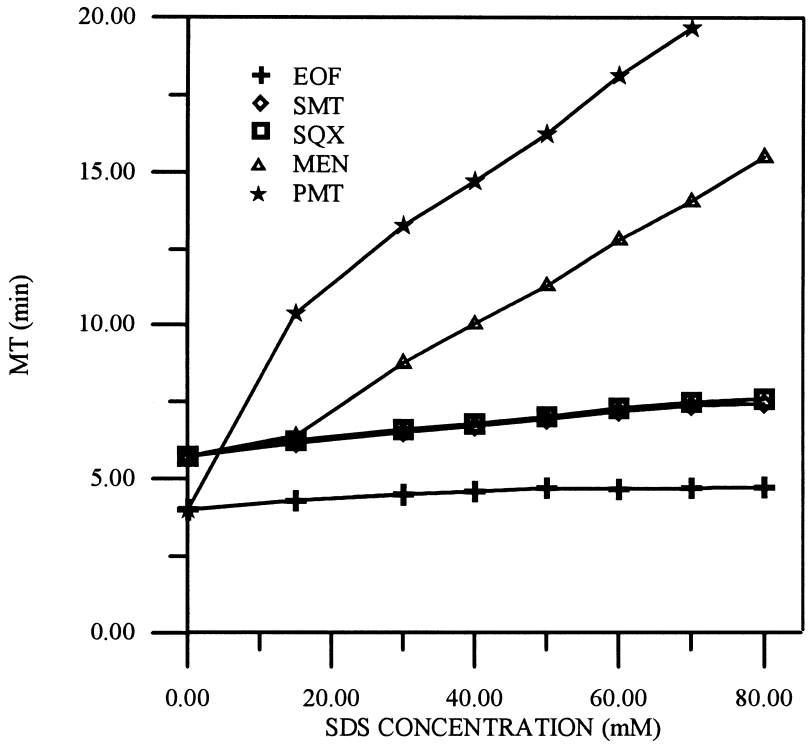


Figure 2. Influence of SDS concentration on migration times.

Influence of SDS

The influence of SDS in the electrolyte on the migration time (MT) is given in Figure 2. The operating potential and temperature were 20 kV and 25ºC, respectively. A SDS concentration range from 0 to 80 mM was taken for this part of the study. The results show that an increasing SDS concentration has virtually no effect on the SMT and on SQX migration times, but it does dramatically in the cases of MEN and PMT. This behaviour can be explained because SMT and SQX are ionized (anionic forms), whereas MEN and PMT are not, so they shall report more interactions with the micelles.

A 40 mM SDS concentration was selected for further experiments since it gave good resolution and high narrow peaks making it easier for integration. The current generated was 38.9 μA and the run time about 15 min.

Influence of Borate Buffer Molarity

The borate buffer molarity was varied from 10 to 40 mM using the experimental conditions mentioned above and its influence upon the MT was studied. A 30 mM concentration was considered as suitable for its good resolution and peak shape, whereas higher concentrations resulted in peak broadening.

Effect of the Temperature

The effect of the temperature on the separation was tested between 20 and 40ºC. According to resolution, run time, and current generated (53.8 µA), 30ºC was selected as suitable.

Influence of the Voltage and Voltage Ramp

The effect of varying the voltage from 10 to 30 kV was investigated under the conditions set out above. A potential of 20 kV yielded the best compromise in terms of run time, current generated (54.1 µA), and linearity between voltage and current. Different voltage ramps, 0.2 s to 0.8 s, were studied and then analyzed in terms of resolution of SQX from SMT, whose respective migration times are so close to each other. The results indicated that when the optimum voltage (20 kV) is reached in 0.4 s, the maximum resolution is provided.

However, in these conditions the run time is too long and a second potential ramp was tested. Several experiences were performed related to this subject, but it was decided to raise the voltage up to 30 kV in a 0.4-second voltage ramp, at t = 5 min, and let the separation end this way. Finally, the optimized conditions for the separation are summarized in Table 1 and the corresponding electropherogram is showed in Figure 3.

Limits of Detection and Quantification

Limits of detection and quantification (LOD and LOQ, respectively) were estimated in accordance to the base line noise method. The base line noise was evaluated by recording the detector response over a period of 10 times the peak width. The LOD was obtained as the sample concentration that caused a peak with a height three-fold the base line noise level and the LOQ was calculated as ten-fold the base-line noise level. Thus, LOD and LOQ were estimated to be 300 µg/L and 1 mg/L, respectively, for each component.

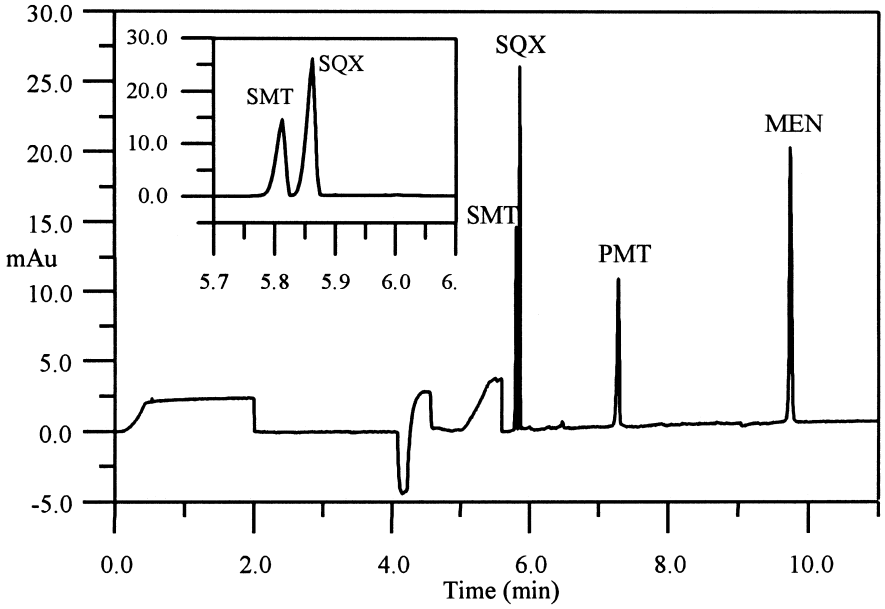


Figure 3. Typical electropherogram under optimized conditions for the separation (30 mg/L SMT, SQX, and MEN and 20 mg/L PMT; λ = 214 nm).

Table 1

Optimized Conditions for the Separation

Capilary	Fused silica (57 cm length x 75 μm inner diameter)
Electrolyte	30 mM borate buffer (pH 9.2) : 40 mM SDS: 6 % Acetonitrile
Temperature	30°C
Voltage ramp	20 kV from 0 to 5 min. and 30 kV till the end
Detector	Diode array
Window	800 x 100 μm

Table 2

Linear Regression Calibration Curves

Component	Linear Regression Curve	r^2	Linearity Range (mg/L)
SMT	A = -17 (± 89) + 120.7 (± 1.9) conc (mg/L)	0.9998	2 - 80
SQX	A = 52 (± 190) + 164.4 (± 4.2) conc (mg/L)	0.9995	2 - 80
MEN	A = -94 (± 122) + 183.3 (± 2.7) conc (mg/L)	0.9998	2 - 80
PMT	A = -56 (± 149) + 229.9 (± 5.3) conc (mg/L)	0.9997	2 - 45

Linearity Range and Calibration Curves

The selected parameter for the integration was the corrected area, measured at 263, 252, 250, and 210 nm for SMT, SQX, MEN, and PMT, respectively, in order to obtain very low standard deviations in the in-a-day and day-to-day reproducibility measures (RSD about 1.3 %).

Since the LOQ was calculated around 1 mg/L of each component, the calibration curves were proposed from these concentration levels on. Thus, the resulting calibration curves are shown in Table 2.

In these calibration curves, a linear relationship between concentration and corrected area for each component can be seen and, also, they all have intercepts considered as negligible if tested by the t of Student ($\alpha = 0.05$).

The middle point of the calibration curve was selected to study the influence of the injection time on the corrected area, from 2 to 10 s, resulting in similar linearity conditions that were already provided.

APPLICATION

The proposed method was tested for determining some binary and ternary mixtures of the named substances in six commercial preparations used in the veterinary practice.

Due to the high viscosity of the commercial products, it is not possible to take an exact volume by using a pipette. So, we decided to always take 10 mL of the samples with a 10 mL volummetric flask and also calculate, by weighing

Table 3

Application Results (g/L)*

			Found		Recovery (%)	
		Claimed	**MECC**	**HPLC**	**MECC**	**HPLC**
COCCIAMIN	SQX	50	28.7	28.7	57.5	57.5
	PMT	15	8.3	8.8	55.3	58.7
COCICHEMICAL	SQX	50	29.6	30.3	59.1	60.5
	PMT	15	---	---	---	---
COCCILINA	SQX	3.9	3.13	3.20	80.3	82.1
	PMT	1.3	0.89	0.93	68.2	71.4
	SMT	0	0.76	0.81	---	---
COCCIREX	SQX	44	42.0	44.3	95.4	100.6
	PMT	12	10.2	11.5	85.2	95.8
COCCIVET	SQX	36	32.3	31.7	89.8	88.2
	PMT	9	8.3	9.0	92.1	100.3
	MEN	50	---	---	---	---
QUINOXIPRA-P	SQX	50	48.7	49.3	97.4	98.7
	PMT	15	14.2	14.3	94.6	95.6

* The concentration units in Coccillina are g/100 g.

the difference, the sample weigh. Thus, it is possible to give results in weigh:weigh or weigh:volume units, depending on the way used in each commercial. Then, 10 mL were diluted with ethanol into a 250 mL volummetric flask, where different volumes were taken from, according to the expected drug and/or vitamin levels in each case, and diluted with water in 25 mL volummetric flasks, adding ethanol till 14% existing in the calibration and standard solutions. A HPLC method was developed to confirm the results obtained in MECC. The same compounds were determined by HPLC using as mobile phase 0.1 M NaH_2PO_4 (pH 3.0)-acetonitrile (7:3 to 4:6 gradient) and a Kromasil C_{18} as analytical column. Flow rate and injection volume were 1.5 mL/min 20 μL, respectively. A diode-array deteector was used as well. The amounts and recoveries found (summarized in Table 3) are worth being commented:

i) During the analysis procedure of Cocciamin and Cocichemical, no precipitate or thickness were observed, so their low recoveries are just due to their own SQX and PMT contents.

ii) MEN and PMT are not quantified in Coccivet and Cocichemical, respectively. This is because they are not present as the pure components. Thus, the respective laboratories where these commercials come from were consulted about. MEN is added in its sodium bisulphite form, but no answer has been received yet about the way PMT is included in Cocichemical.

iii) SMT was found in Coccilina, but it is not shown in claimed levels. The presence of SMT in Coccilina was confirmed by overlapping the sample and standard spectra and by means of the standard addition method as well.

CONCLUSION

The results obtained show that MECC is a valuable method for the determination of sulfonamides and their associated compounds. Assay and repeatability results are comparable to those obtained with the HPLC method and comply with the requirements of drug quality control. MECC is suitable for routine use and offers simplicity of operation, flexibility, and low cost as advantages. This method has been succesfully applied to determine SMT, SQX, MEN, and PMT in commercials used in the veterinary practice.

The results, confirmed by HPLC, show that in some products, the actual composition is not in agreement with the claimed levels and/or composition.

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