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Electroosmotic flow variations caused by the volatility of buffer components: diagnosis and therapy ☆

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Abstract

In order to separate a polar amine pharmaceutical and its potential impurities, a micellar electrokinetic chromatography method was developed. The main compound and 11 other substances were completely separated using a 20 m*M* Tris buffer, pH 8.0, containing 50 m*M* sodium dodecylsulfate (SDS) and 24% (v/v) acetonitrile. However, a strong, continuous reduction in the EOF occurred and quantification was not possible. The EOF reproducibility could not be improved by suitable rinsing procedures. Surface effects or interactions did not cause the EOF changes, but the evaporation of acetonitrile was identified as the major source for EOF instability. However, a high concentration of acetonitrile was decisive for selectivity. Thus a reliable protection against the evaporation of this buffer constituent had to be found. Paraffin and various silicon oils were tested as covering film. In order to quickly test buffer systems if the evaporation of electrophoresis solutions is acceptable, an alternative experimental design without doing CE experiments had to be found. Electrical conductivity was chosen as parameter, because it can be determined simply and fast. The buffers under investigation were placed in a 50-ml beaker with a magnetic stirring rod, placed on a magnetic stirrer. The buffer solution was kept in motion at 120 rev./min. The beaker was covered around the measuring head with laboratory film and in addition to this with paraffin or silicon oil. An acetonitrile content up to 10% (v/v) was acceptable if a coverage was used. The various cover liquids had a similar effect. A content of 15% (v/v) already increased the evaporation effect significantly. Higher acetonitrile contents are not acceptable. A buffer similar to the originally transferred method, 20 m*M* Tris (pH 8.0), 50 m*M* SDS containing 10% (v/v) acetonitrile as well as 10% (v/v) isopropanol showed an acceptably low evaporation in the conductivity experiments. With this buffer, a stable EOF was also obtained. Conductivity measurements are generally applicable to quickly test buffers that contain organic solvents.

Keywords: Electroosmotic flow; Buffer composition

1. Introduction

A micellar electrokinetic chromatography (MEKC) method was developed in order to separate a polar amine pharmaceutical and its potential impurities. The main compound and 11 other substances were completely separated using a 20 m*M* Tris buffer, pH 8.0, containing 50 m*M* sodium dodecylsulfate (SDS) and 24% (v/v) acetonitrile. However, a strong, continuous reduction in the migration times occurred. For the main compound, the migration time increased from 26 min to more

☆Dedicated to Professor Dr. Gotthard Wurm on the occasion of his 65th birthday.

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than 1 h during nine subsequent runs. Quantification was not possible under these circumstances. The changes in the migration times were due to a continuous decrease of the electroosmotic flow (EOF).

A reproducible EOF is always required to obtain reproducible migration times and peak areas in CE [1]. However, in practice, changes in EOF are frequently observed. For example, using an MEKC method (20 m*M* Tris buffer, pH 8.0, containing 50 m*M* SDS and 24% (v/v) acetonitrile; Section 2.1) to separate a polar amine pharmaceutical and its potential impurities, the migration time of the EOF peak increased from 4.93 to 5.06 and to 5.23 min in the 2nd, 4th and 6th run of one series. At the same time the migration times of all analytes increased, e.g. from 28.9 to 33.5 and to 36.7 min for the main compound (2nd, 4th and 6th run, respectively). The higher the migration time of an analyte peak, the stronger its migration times were affected. During the measurement series, t_{EOF} later increased to more than 6 min, thus the migration time for the main peak even exceeded 1 h.

Even minor changes in EOF can have enormous, cantilever-like, effects on the migration time. Especially later eluting peaks are often strongly affected. Their gross mobility μ_{gross} is already small and if it comes close to zero, the migration time t_M goes towards infinity. In MEKC, the EOF influences both the analysis time and the elution window.

In contrast to chromatography, in CE molecules do not pass the detector cell with a constant velocity. As molecules migrate slower, they will stay longer in the detection cell and will give a detector response for a longer time. Bigger peak areas result. This effect must not be neglected. If, for example, racemic mixtures of enantiomers are analyzed, the peak areas are not equal but the later eluting peak is slightly larger [2,3]. The effect of changing analyte mobility can partly be compensated by the use of corrected peak areas (peak area/migration time). These should be proportional to the sample concentration even if the mobility of an analyte changes from run to run (Ref. [1] and references cited therein).

Using corrected peak areas, it is assumed that the mobility remains constant at least during one run. However, the EOF and thus the mobility can already change during a separation by adsorption and surface changes of the capillary [4,5]. Therefore, sometimes it will not be sufficient to use corrected areas, especially if strong changes in t_M occur. Thus it is necessary to reduce migration time variations as much as possible [6]. A stable EOF is also necessary to estimate correctly sample mobility and sample amount. Therefore the electroosmotic flow is a crucial factor to be controlled for validated CE methods.

2. Experimental

2.1. Capillary electrophoresis

The CE experiments were carried out with a 40 cm (capillary inlet to detection window, 48.5 cm total length) fused-silica capillary, 50 μm I.D. using a Tris buffer 20 m*M*, pH 8.0, containing 50 m*M* SDS and 24% (v/v) acetonitrile. This buffer is prepared by dissolving 2.4228 g of Tris in ~650 ml water (HPLC quality). The pH of this solution is adjusted to 8.00 using 0.1 *M* HCl. Then 14.419 g of sodium dodecylsulfate are added and dissolved; 240.0 ml of acetonitrile (HPLC quality) are added. Finally this solution is made up to 1000.0 ml with water. All buffer constituents were from Fluka (Germany).

Prior to each run, the capillary was rinsed for 3 min with HPCE-grade water and 3 min with buffer. A voltage of 20 kV was applied, resulting in a current of ~33 μA. The sample is injected by applying a pressure difference of 50 mbar for 3 s. The detection wavelength was set to 200 nm. The temperature was set to 30 °C. Prior to first use, the capillaries were conditioned by rinsing with 0.1 *M* sodium hydroxide for 30 min. Then it is filled with the buffer and equilibrated for at least 2 h.

2.2. Conductivity measurements

The respective buffers were placed in a 50 ml beaker with a magnetic stirring rod, placed on a magnetic stirrer which was covered by an ~2 cm thick styrofoam plate (Fig. 2). The buffer solution was kept in motion at 120 rev./min. The beaker was covered with laboratory film around the measuring head and in addition to this with paraffin or silicon

oil in some experiments, in order to minimise evaporation. The conductivity was measured at 15 min using a KLE 315 instrument (WTW, Wissenschaftliche Technische Werke, Weilheim, Germany). The temperature in the buffer was kept constant at 20±0.2 °C.

2.3. Liquid coverages

Several experiments were performed using a liquid film of ~3 mm thickness. As cover liquids, paraffinum subliquidum (pharmacopoeia quality, Caelo, Hilden, Germany) and several silicon oils (DC200 0.937, As 4 0.962 and DC200 0.963 from Fluka, Taufkirchen, Germany) were used.

3. Results and discussion

3.1. Parameters that influence the EOF

The walls of untreated fused-silica capillaries are negatively charged, because the silanol groups are partly deprotonated [7,8]. The EOF arises from cations, which are attracted by the capillary wall. A concentration gradient of positive charge close to the capillary walls results from the competition of electrostatic attraction and diffusion, known as ζ potential. The solvatized cations migrate towards the cathode taking along the buffer solution by frictional forces [9,10].

The EOF mobility μ_{EOF} can be estimated using Eq. (1) [11]:

$$\mu_{EOF} = \frac{\zeta\varepsilon}{4\pi\eta} \quad (1)$$

η is the viscosity of the electrophoresis solution and ε its dielectric constant. When working with lower concentrations of organic solvents, changes in the dielectric constant are minor. Hence the two major parameters that influence the EOF are the ζ potential and the viscosity η (Fig. 1). The ζ potential is influenced by the buffer concentration and the surface charge. However, the buffer concentration can easily be kept constant and is no reason for EOF instability. There are several reasons for changes in surface charge which subsequently influence the EOF, but most of them can be easily controlled as well, such as buffer pH and external radial fields. The hydrolysis of capillary coatings is frequently reported to increase the EOF, but no coated capillaries were used here. The EOF is usually well reproducible for a pH<2.5 and in the pH range between 8 and 10 [1], thus the buffer pH of the system under investigation was unlikely to be critical. If pH-related difficulties are found, longer preconditioning times (e.g. 2–10 h) with higher NaOH concentrations (e.g. 1 *M*) are recommended

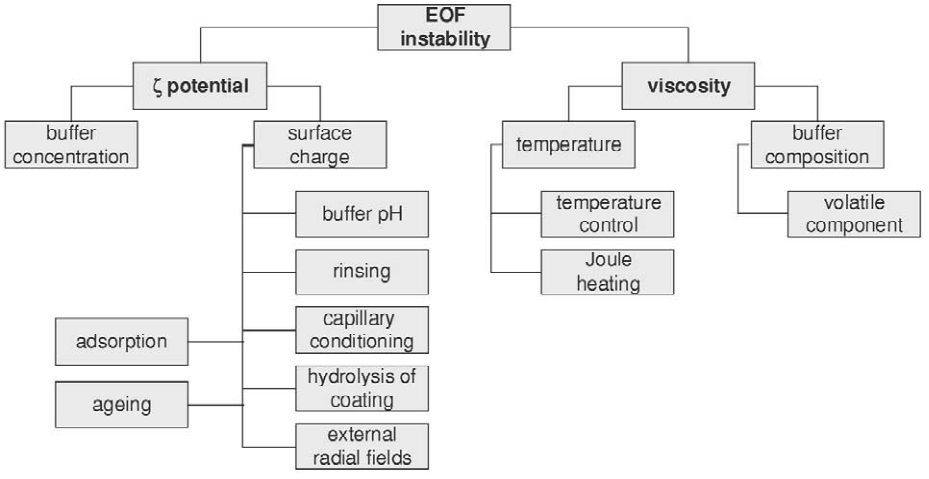


Fig. 1. Possible reasons for EOF instability.

[12,13]. These longer preconditioning times did not influence the EOF in this case, though.

The EOF is often strongly influenced by the adsorption of sample molecules at the capillary walls (e.g. Refs. [1,14–17]). However, in the case under consideration, the analytes were small molecules with few binding sites; permanent adsorption of these was very unlikely. If adsorptions were the reason for the EOF decrease, these could be removed by suitable rinsing protocols. Rinsing regimens are also useful in other cases, when changes to slower EOF velocities are found. Hypotheses about this effect consider gel formation, weathering, aging, or trace constituents in the buffer as possible reasons [1]. When the capillary is rinsed with sodium hydroxide followed by water and then fresh buffer, e.g. once a day, the reproducibility of migration times has been significantly improved [18–22]. However, numerous rinsing protocols, including the use of sodium hydroxide and running buffer with SDS and various organic solvents had no positive effect in this case.

The viscosity depends on the temperature and on the content of polymers or organic solvents, which are frequently used as additives in CE. The EOF is inversely proportional to viscosity [23]. Thermal effects may be observed using high electrical power. As a rule of thumb, Joule heating is uncritical using a power below 2.5 W/m for 25 μm capillaries and below 5 W/m for a 50 μm capillary provided that the instrument is properly thermostated [1]. In the discussed example however the power was well below 1.5 W/m.

3.2. The evaporation of acetonitrile: the major source for EOF instability

In the case under investigation, the evaporation of the organic modifier acetonitrile was identified as the main reason for the EOF decrease. EOF changes through solvent evaporation have not been reported before despite the frequent use of solvents in buffers for CE. However, in another study the evaporation of acetonitrile perceptibly influenced the injection volume [24,25].

It was not possible to substitute acetonitrile for another solvent because of its unique position in Snyder's selectivity triangle [26]. Alkyl nitriles with longer chain length also show different dipole–dipole interactions.

The use of various vial caps could not prevent evaporation, because the caps leaked through slits or holes that were necessary to provide the contact of the capillary and the electrode to the buffer. Buffer cooling in the sample tray was also discussed to reduce evaporation. However, sufficient cooling capacity must be provided.

Instead of using vial caps, evaporation can better be avoided by finding liquids which are lighter than the buffer and do not solve any of the buffer constituents (like that demonstrated in Ref. [27]). Mineral oil (paraffin) proved to be a very useful substance for covering the buffer vials in order to prevent both oxidation and evaporation [24,25,27]. The cover liquid is reliably excluded from the capillary by surface tension effects, current breakdowns or signals from mineral oil drops were never observed. Using this cover, evaporation was no longer observed, and reproducibility of 0.53% RSD for relative peak areas ($n=60$) was obtained [24]. In the works mentioned, no influence of the cover liquid on selectivity was observed.

In order to transfer this concept, a mineral oil coverage was tried here as well. Some improvements could be noted for migration time reproducibility. Like in earlier works, no influence of the coverage on the selectivity was observed.

However, the EOF migration times still increased far too strongly. Due to the high content of organic modifier, the coverage was not efficient enough. The role of SDS micelles as potential transporters through the coverage was considered, but buffers without SDS containing 24% v/v acetonitrile showed the same increase in EOF times despite the paraffin coverage.

3.3. A method to quickly evaluate solvent evaporation

From these findings it was concluded that only a certain amount of organic modifier is acceptable. However, it would be very time-consuming to determine this limit by CE measurement series, considering that the limit should be dependent on the used solvent and possibly on other buffer constituents. Moreover, it is interesting to study various cover

liquids. Thus a fast alternative to evaluate solvent evaporation was needed.

A slightly increasing current was observed during a series of runs for a method to measure insulin concentrations [24,25]. Apparently the evaporation of organic solvents measurably changes the electrical conductivity. This test parameter can be determined simply and fast. Moreover, a straightforward experimental set-up can be used (see Section 2).

In preliminary experiments, strong changes in conductivity after a few minutes were found, which could be explained by a slight temperature increase due to the warming of the plate of the magnetic stirrer. Therefore, all conductivity data reported here were measured insulating the plate of the magnetic stirrer by an ~2 cm thick styrofoam plate (Fig. 2). After this insulation, the measured temperature was constant at 20±0.2 °C in the following experiments.

The conductivity massively rose within the first 4 h for the buffer used for the CE experiments described above (see Section 1). When the buffer was covered by a paraffin layer, the increase in conductivity was slower, but still strong. When a buffer system with 10% methanol was investigated, which was known to give a stable EOF, no significant increase in conductivity was found.

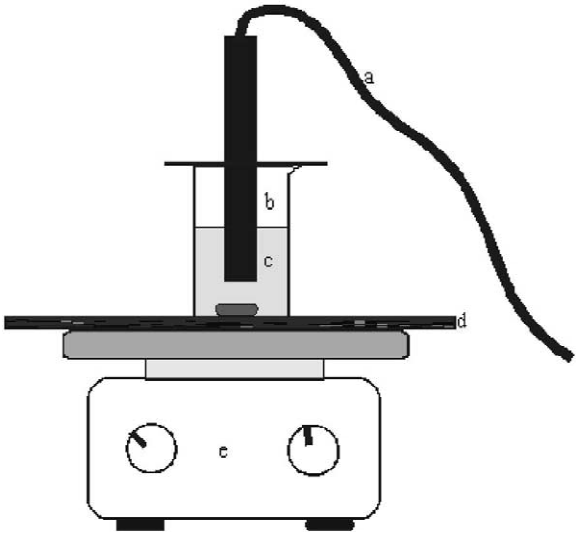


Fig. 2. Set-up for conductivity measurements (Section 2.2). (a) Measuring head, (b) position of the covering liquids, (c) buffer solution, (d) styrofoam plate, (e) magnetic stirrer.

The conductivity measurements were completely consistent with the CE results but showed a much better time resolution (Fig. 3A–C). Thus they are very well suitable to characterize evaporation properties.

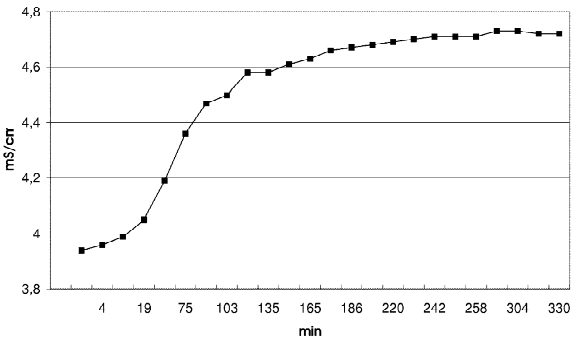
3.4. Results from conductivity measurements

The paraffin was saturated with buffer components before use, because it was shown that the reproducibility of a measuring series improved a lot when the buffer solution was covered with a saturated paraffin solution [28]. This improvement was also found here, but a distinct increase in conductivity still remained.

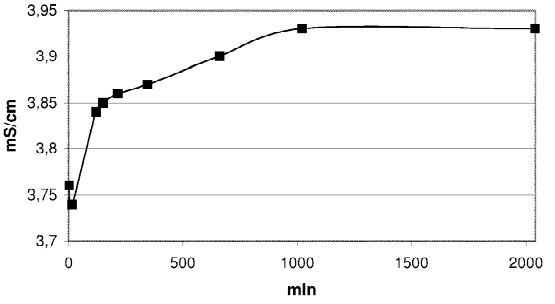
A buffer of 20 m*M* Tris (pH 8.0) without SDS, but with the same concentration of acetonitrile (24%, v/v) was tested, obtaining the same strong increase in conductivity. Thus it was shown that the formation of micelles does not have any effect on the transport of acetonitrile through the paraffin layer (Fig. 4).

Next it was tried if other liquids were more efficient than paraffin to avoid evaporation. Five ml of paraffin or silicon oil were used, corresponding to liquid film thickness of ~3 mm. Liquids that do not mix with water had to be used. Moreover, their densities had to be significantly lower than that of water to avoid the formation of emulsions. Experiments were performed with silicon oil As 4 (δ = 0.962 g cm^{-3}) and two silicon oil DC200 species with different densities (δ = 0.937 and 0.963 g cm^{-3}, respectively). The cover liquids were buffer-saturated before the measurements. All gave essentially the same results like paraffin: significant but insufficient protection.

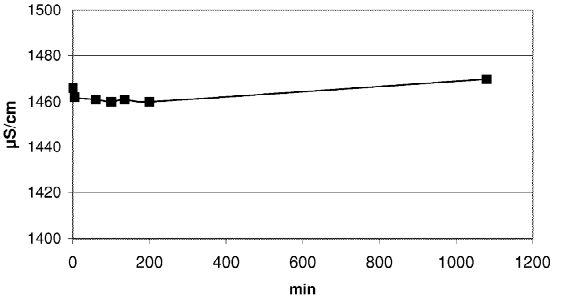
The described test system allowed for the quick check of numerous buffer compositions. The stepwise reduction of the acetonitrile content showed that there is a threshold from which evaporation effects become critical. An acetonitrile content up to 10% (v/v) was acceptable, if the vials were properly covered. A content of 15% (v/v) already increased the evaporation effect significantly. The evaporation cannot be effectively controlled up to now, when higher acetonitrile contents are used. If 15% (v/v) acetonitrile is already critical, non-aqueous CE meth-



(A)



(B)



(C)

Fig. 3. Changes in conductivity with time demonstrating consistency of results from CE and conductivity measurements. (A) Tris buffer 20 m*M*, pH 8.0, containing 50 m*M* SDS and 24% (v/v) acetonitrile (see Section 2). Fast increase in conductivity from evaporation of acetonitrile, confirming this is the reason for the increase in EOF. (B) Like (A), but using an additional silicon oil D 200 (0.937 g/ml) coverage of 3 mm thickness. Increase in conductivity only slightly reduced, confirming that this coverage is insufficient. (C) Acetate buffer containing 10% (v/v) methanol, used for Flubilar analysis [12]. Here no significant change in conductivity was noted, corresponding to insignificant solvent evaporation and a very stable EOF over time using this buffer system.

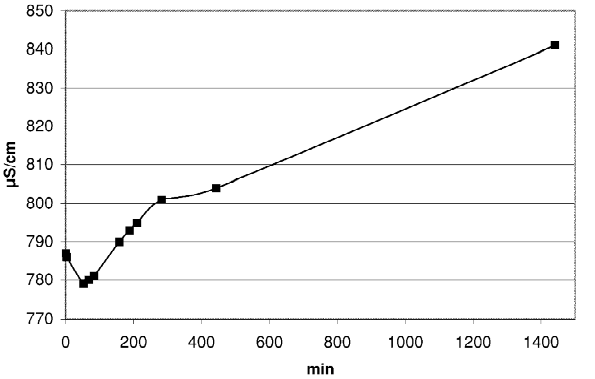


Fig. 4. Tris buffer 20 m*M*, pH 8.0, containing 24% (v/v) acetonitrile (but no SDS), covered with buffer-saturated paraffin. Significant reduction in acetonitrile evaporation (compare Fig. 3A), but still no stable conductivity over several hours.

ods must be considered quite problematic in terms of EOF reproducibility.

Interestingly, these acetonitrile concentrations can be used when another solvent is added. When 10% methanol and 10% acetonitrile were used, the conductivity remained stable; using 10% methanol and 15% acetonitrile, the conductivity increase is the same as for only 15% acetonitrile (Fig. 5, Table 1).

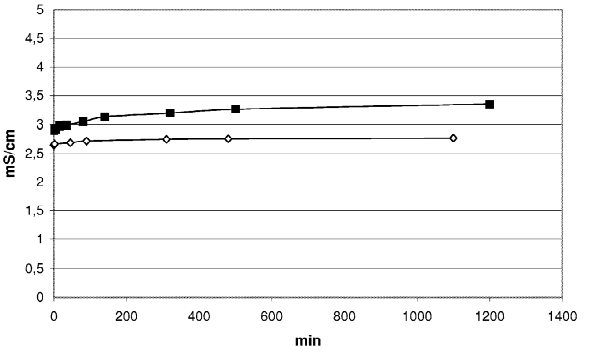


Fig. 5. Critical threshold for the acetonitrile concentration. Tris buffers 20 m*M*, pH 8.0, containing 10% (v/v) methanol and acetonitrile, 10% (open rhombi) or 15% (v/v) (filled squares), respectively, using an additional silicon oil D 200 (0.937 g/ml) coverage of 3 mm thickness. Note that the absolute conductivity depends on the precise position of the measuring head, which was slightly different in both series. However, the relative change can be well compared. An acetonitrile content up to 10% (v/v) was acceptable, if the vials were properly covered. A content of 15% (v/v) already increased the evaporation effect significantly.

Table 1
Limit for acceptable acetonitrile concentrations in CE buffers

Acetonitrile (%, v/v)	Comment	Ref.
10	Suitable	Fig. 5
12.8	Suitable	[1,28]
15	Borderline	Fig. 5
24	Not acceptable	Buffer from Section 2.1

4. Conclusions

The evaporation of acetonitrile was identified as the major source for EOF instability. In order to test if a certain content of an organic modifier was acceptable, conductivity measurements have been established. Effective heat insulation was found important in order to obtain reproducible results. This test system is generally applicable to quickly test buffers that contain organic solvents. Cover liquids significantly decrease effects from the evaporation of organic solvents. Various cover liquids showed very similar properties. The saturation of the cover liquids with buffer significantly improved the results.

An acetonitrile content up to 10% (v/v) was acceptable using an appropriate cover liquid. A content of 15% (v/v) already increased the evaporation effect significantly. Higher acetonitrile contents are not acceptable. If 15% (v/v) acetonitrile is already critical, non-aqueous CE methods must be considered quite problematic in terms of EOF reproducibility. A buffer similar to the originally used method, 20 m*M* Tris (pH 8.0), 50 m*M* SDS containing 10% (v/v) acetonitrile as well as 10% (v/v) isopropanol showed an acceptably low evaporation. With this buffer, a stable EOF was also obtained. If higher contents of organic solvents shall be used, a more effective prevention of evaporation, e.g. special vials in combination with liquid film coverage, has still to be found.

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