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Journal of Chromatography A, 735 (1996) 227–235

JOURNAL OF CHROMATOGRAPHY A

Complementary use of capillary zone electrophoresis and micellar electrokinetic capillary chromatography for mutual confirmation of results in forensic drug analysis

F. Tagliaro[a,*], F.P. Smith[b], S. Turrina[a], V. Equisetto[a], M. Marigo[a]

[a]*Institute of Forensic Medicine, University of Verona, 37134 Verona, Italy*

[b]*Graduate Program in Forensic Science, Department of Criminal Justice, The University of Alabama at Birmingham, Birmingham, AL 35294-2060, USA*

Abstract

The purpose of this work was to compare different CE separation modes, namely capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC), for the analysis of drugs of forensic interest in order to assess the mutual degree of independence and consequently the possibility of complementary use for mutual confirmation of results. A panel of drugs including caffeine, morphine, barbital, pentobarbital, codeine, nalorphine, lidocaine, procaine, heroin, flunitrazepam, acetylcodeine, papaverine, amphetamine, narcotine, cocaine, diazepam, tetracaine, narceine, 6-monoacetylmorphine acetylcodeine and thebaine, were separated according to a MECC and two CZE methods. The MECC separation was carried out in a bare silica capillary (50 μm I.D.) with a buffer composed of 25 m*M* borate (pH 9.24)–20% methanol–100 m*M* sodium dodecyl sulphate; the applied voltage was 20 kV. The first CZE method (CZE1) was carried out in 50 m*M* phosphate buffer (pH 2.35) at 20 kV with a bare silica capillary (50 μm I.D.), and the second (CZE2) with 50 m*M* borate (pH 9.24) at 12 kV with the same capillary. The three methods were effective in the separation of the test drug mixture, but MECC was the only able to resolve all the components. Relative (to flunitrazepam) migration time RSDs ranged from 0.3 to 2.8% for MECC, from 0.1 to 1.4% for CZE1 and from <0.1 to 0.5% for CZE2. The separation patterns (migration order) of the three methods were compared with Spearman's test and with principal component analysis. CZE1 and CZE2 were significantly and directly correlated ($r = 0.749$, $p < 0.002$), whereas MECC and CZE2 were also significantly, but inversely correlated ($r = -0.865$, $p < 0.001$). MECC and CZE1 (limitedly to the basic drugs) appeared non-correlated ($r = -0.131$, $p = 0.630$) and therefore the two techniques are suitable for combined use to increase the discriminatory power.

Keywords: Forensic analysis; Drugs; Capillary zone electrophoresis; Micellar electrokinetic capillary chromatography; Capillary electrophoresis

1. Introduction

Capillary electrophoresis (CE) is probably the most rapidly growing analytical technique that has appeared in the recent years. Its application soon extended well over the subjects typical of slab gel electrophoresis (e.g., biopolymer analysis), finding a very fertile field in pharmaceutical drug analysis, as is evident from a large and steadily expanding body of literature [1]. This

* Corresponding author.

0021-9673/96/$15.00
SSDI 0021-9673(95)01311-3

has prompted also the introduction of CE into analytical toxicology for the analysis of illicit or controlled drugs either in illicit preparations or in body fluids and tissues [2–4].

The advantages of CE are related to the excellent separation efficiency, the high mass sensitivity, the minimal use of samples and solvents and the possibility of using different direct and indirect detection systems (including mass spectrometry). However, probably, the main innovation brought by CE in analytical toxicology is represented by the peculiar separation principles on which it is based, which are different from those of the analytical techniques currently applied in this field. Hence, CE for drug analysis has proved highly complementary with traditional chromatographic approaches, i.e., electrophoresis and chromatography based on non-correlated physical-chemical principles of separation.

This is important because a generally accepted requirement in forensic science is that, to achieve legal defensibility, experimental results have to be obtained and confirmed by at least two independent methods, based on different analytical mechanisms with comparable sensitivity. This is traditionally achieved by coupling, e.g., immunoassay and chromatography, two different chromatographic methods or chromatography and spectrometry (mainly mass spectrometry).

In particular, micellar electrokinetic capillary chromatography (MECC) has been successfully compared with gas and liquid chromatography for the analysis of illicit heroin and cocaine substances [5–8] and controlled anabolic steroids [9]. In the studies cited, MECC and chromatography gave very different separation patterns, thus proving to be "orthogonal" techniques.

However, capillary zone electrophoresis (CZE) has also been used for the analysis of water ionizable abused substances, showing excellent resolution and efficiency not only on test drug mixtures [10], but also in biological fluids and hair [11].

Since CZE is based on relatively pure electrophoretic separation principles, whereas MECC integrates also reversed-phase chromatography-like mechanisms, they are in principle "orthogonal enough" to postulate complementarity of the two CE separation modes. With this aim, it is important to demonstrate in practice that CZE and MECC separations are not correlated with each other, giving rise to different separations patterns when specifically applied to analytes of interest.

To the best of our knowledge, CZE and MECC have never been studied from this point of view in the separation of drugs of forensic interest. This was the purpose of the present work, in which the separation patterns of a panel of forensic substances (including heroin, heroin metabolites, impurities and adulterants and other illicit and/or abused drugs) obtained by means of MECC and CZE were compared to infer the degree of mutual correlation.

2. Experimental

2.1. Materials

All solvents of HPLC grade and buffer salts of analytical-reagent grade were purchased from Carlo Erba (Milan, Italy). Analytical-reagent grade sodium dodecyl sulphate (SDS) was provided by Merck (Darmstadt, Germany). The running buffers were filtered and degassed under vacuum through 0.45-μm disposable PTFE filters before use (Bio-Rad, Richmond, CA, USA).

The standard substances analysed (caffeine, morphine, barbital, pentobarbital, codeine, nalorphine, lidocaine, procaine, heroin, flunitrazepam, acetylcodeine, papaverine, amphetamine, narcotine, cocaine, diazepam and tetracaine) were obtained from Sigma (St. Louis, MO, USA), narceine, 6-monoacetylmorphine (MAM) and acetylcodeine were obtained from Salars (Como, Italy) and thebaine from Carlo Erba. Stock standard solutions were prepared in methanol at a concentration of 1 mg/ml each and stored at −20°C. Weekly, 1-ml working aliquots of all the standards were prepared in distilled water at suitable concentrations (ranging from 1 to 50 μg/ml) from the stock standard solutions and stored refrigerated at 4°C.

2.2. Instrumentation

A manual capillary electropherograph (Model 3850; ISCO, Lincoln, NE, USA) was used in all experiments. Detection was accomplished "in-column" by means of a UV detector operated at the wavelength of 200 nm, with a full-scale range of 16 mAU. Injection was by a split-flow injector with a splitting ratio of about 1:800, which was loaded by an HPLC syringe usually delivering 5 μl of sample. Bare fused-silica capillaries (J&W Scientific, Folsom, CA, USA) with I.D. of 50 μm and a total length of about 55 cm (35 cm to the detector) were used. All the separations were accomplished at a constant potential, which was chosen to keep the background current below 60–80 μA.

In addition, preliminary reproducibility tests were carried out using an HP 3D CE Model G1600A fully automated instrument (with column thermostating), fitted with a diode-array UV detector, kindly lent by Dr. D.N. Heiger (Hewlett Packard, Wilmington, DE, USA).

2.3. Methods

Basically, mixtures of the drugs were subjected to both MECC and CZE separations; CZE, in particular, was carried out under alkaline (pH 9.24) and acidic (pH 2.35) conditions. Briefly the analytical conditions of the different experiments were as follows:

MECC: capillary, bare fused silica, I.D. 50 μm, total length 55 cm (35 cm to the detector); buffer, 25 m*M* borate (pH 9.24)–methanol (80:20) containing 100 m*M* SDS; potential, 20 kV.

CZE1: capillary, bare fused silica, I.D. 50 μm, total length 55 cm (35 cm to the detector); buffer, 50 m*M* phosphate (pH 2.35); potential, 20 kV.

CZE2: capillary, bare fused silica, I.D. 50 μm, total length 55 cm (35 cm to the detector); buffer 50 m*M* borate (pH 9.24); potential, 12 kV.

In any experimental mode, capillary conditioning was carried out by flushing with running buffer for about 5 min; periodically, the capillary was washed with 0.1 *M* NaOH and extensively conditioned with buffer.

2.4. Experimental design

All the standard drugs were first analysed individually and then simultaneously by each CE analytical mode. Average absolute and relative (to flunitrazepam) migration times ± relative standard deviation (R.S.D.) were calculated on the basis of six injections repeated on the same day.

Day-to-day evaluation of variability was omitted with the manual instrument, because it was beyond the scope of this work and also because the reproducibility of the capillary conditioning was too dependent on variable factors, related to the manual operation mode.

A more complete, but still preliminary, evaluation of the variability of CZE and MECC, comprising intra- and inter-day R.S.D., was carried out with the HP 3D CE automated instrument (five series of injections per day carried out on four different days).

The patterns of the MECC and the two CZE separations were then statistically compared to investigate the degree of mutual correlation using Spearman's correlation test and principal component analysis, performed with the Statgraphics (STSC) software package.

3. Results and discussion

3.1. MECC

Since the first paper by Weinberger and Lurie [5], MECC has widely been shown to provide efficient separations of forensic drugs (acidic, basic and neutral) and the present data confirm these previous findings. In fact, among the CE methods tested, MECC was the only one able to resolve all the 20 drugs tested within about 45 min, with an efficiency in excess of 80 000–90 000 plates, even for the late-eluting peaks (Fig. 1). The separation pattern is substantially in agreement with those reported by Weinberger and Lurie [5] and Krogh et al. [12], who also used MECC, but at slightly different pHs and with a different organic modifier (acetonitrile instead of methanol).

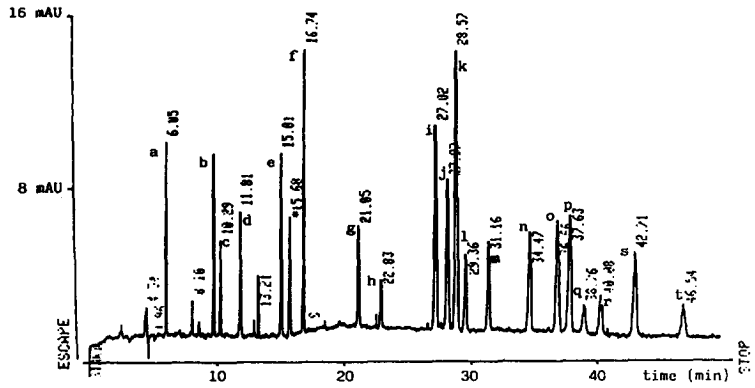

Fig. 1. Typical example of MECC separation of the components of the dug test mixture. Buffer: 25 m*M* borate (pH 9.24)–20% methanol–100 m*M* SDS. For analytical conditions, see text. For peak identification, see Table 1.

Acidic analytes negatively ionized at the buffer pH (9.24) (and caffeine) eluted first, showing a negligible retarding effect by the SDS micelles, and basic compounds followed. No specific attempt was made to correlate migration times with hydrophobicity, but it is clear that congeners with hydrophobic substitutions, such as morphine, MAM and heroin, migrate according to the increasing degree of hydrophobicity.

The reproducibility of absolute migration times was characterized by relatively high R.S.D. values, ranging from 2.0 to 8.6%, because of the variability in conditioning of the capillary by manual flushing, resulting in poor reproducibility of the electroosmotic flow. For this reason, migration times were normalized on the basis of flunitrazepam, thus achieving a substantial improvement in R.S.D.s, which ranged from 0.3 to 2.8% (Table 1).

Since reproducibility in migration times is fundamental for identification purposes, this parameter was verified also with the automated HP 3D CE instrument, fitted with sophisticated control of the capillary temperature. Under these conditions, the precision was much better, showing intra-day R.S.D.s of the relative migration times in the range 0.3–1.5% and day-to-day R.S.D.s ranging from 0.8 to 2.0% (the R.S.D.s of absolute migration times were only slightly higher, indicating excellent control of the electroosmotic flow).

The analytical sensitivity (signal-to-noise ratio = 3) ranged between about 1 and 9 μg/ml, depending on the individual molar absorptivity of the substances.

The study of linearity, quantitative precision and accuracy was beyond the scope of this work and hence was omitted.

3.2. CZE1

CZE at acidic pH, as already demonstrated by Chee and Wan [10], proved suitable for the analysis of basic drugs whereas, as can be expected theoretically, neutral (caffeine) and acidic (barbiturates) substances co-migrated, having the same mobility as the electroosmotic flow (Fig. 2). At the pH of the running buffer (2.35) the basic drugs of the test mixture were well separated, with only three unresolved peaks (papaverine, heroin and narcotine). The basic analytes, all having almost the same net positive charge, migrated substantially according to their molecular mass, with a correlation coefficient (r) = 0.836 ($p < 0.001$). The great majority of the peaks occurred between 8 and 12 min, but the high efficiency of CZE (generally better than 150 000 theoretical plates) allowed their resolution, notwithstanding poor differences in terms of selectivity values (α).

The intra-day R.S.D.s of absolute migration times were clearly better than in MECC, ranging from 0.2 to 1.2%, even with manual operation,

Table 1
MECC analysis of the test drug mixture

Peak[a]	Compound	Migration time [min ± R.S.D. (%)]	Relative migration time ratio to flunitrazepam ± R.S.D. (%)
a	Caffeine	6.05 ± 2.7	0.21 ± 2.7
b	Barbital	9.70 ± 5.0	0.33 ± 2.0
c	Pentobarbital	10.29 ± 4.8	0.35 ± 2.1
d	Morphine	11.81 ± 3.9	0.40 ± 3.2
e	Narceine	15.01 ± 4.3	0.51 ± 2.8
f	MAM	16.74 ± 4.0	0.57 ± 2.8
g	Codeine	21.05 ± 4.6	0.72 ± 2.5
h	Nalorphine	22.83 ± 2.0	0.78 ± 1.1
i	Lidocaine	27.02 ± 5.7	0.92 ± 1.3
j	Procaine	27.97 ± 2.5	0.95 ± 0.3
k	Heroin	28.57 ± 2.4	0.97 ± 0.6
l	Flunitrazepam	29.36 ± 7.0	1
m	Acetylcodeine	31.16 ± 8.6	1.06 ± 1.2
n	Thebaine	34.47 ± 6.6	1.17 ± 0.4
o	Papaverine	36.66 ± 4.0	1.25 ± 1.0
p	Amphetamine	37.63 ± 7.4	1.28 ± 0.5
q	Narcotine	38.76 ± 7.5	1.32 ± 1.2
r	Cocaine	40.08 ± 4.3	1.36 ± 1.2
s	Diazepam	42.71 ± 8.4	1.45 ± 1.6
t	Tetracaine	46.54 ± 7.0	1.58 ± 1.2

[a] See Fig. 1.
Buffer: 25 m*M* borate (pH 9.24)–20% methanol–100 m*M* SDS.

and the R.S.D.s of relative migration times ranged from 0.1 to 1.4% (Table 2).

In the tests with the HP 3D CE instrument, the intra-day R.S.D.s of the absolute migration times were in the range 0.2–0.3% and day-to-day R.S.D.s ranged from 0.9 to 1.0%. The intra-day R.S.D.s of the relative migration times were <0.1% and the day-to-day R.S.D.s were in the range 0.03–0.17%.

The analytical sensitivity, because of the higher efficiency with consequent less peak dilution, was 3–4 times better than in MECC.

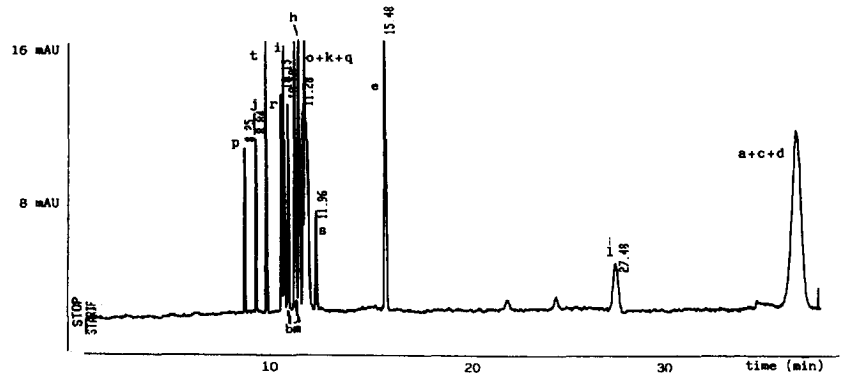

Fig. 2. Typical example of separation of the components of the dug test mixture according to the CZE1 method. Buffer: 50 m*M* phosphate (pH 2.35). For analytical conditions, see text. For peak identification, see Table 2.

Table 2
CZE1 analysis of the test drug mixture

Peak[a]	Compound	Migration time [min ± R.S.D. (%)]	Relative migration time ratio to flunitrazepam ± R.S.D. (%)
p	Amphetamine	8.29 ± 1.2	0.29 ± 0.1
j	Procaine	8.89 ± 0.9	0.31 ± 0.2
t	Tetracaine	9.44 ± 0.9	0.33 ± 0.2
r	Cocaine	10.23 ± 1.1	0.35 ± 0.4
i	Lidocaine	10.36 ± 1.1	0.36 ± 0.1
b	Morphine	10.54 ± 0.8	0.37 ± 0.7
n	Thebaine	10.54 ± 0.8	0.37 ± 0.7
f	MAM	10.98 ± 0.8	0.38 ± 0.7
m	Acetylcodeine	10.98 ± 0.8	0.38 ± 1.4
h	Nalorphine	11.24 ± 0.3	0.39 ± 1.2
o	Papaverine	11.50 ± 1.2	0.40 ± 0.1
k	Heroin	11.50 ± 1.2	0.40 ± 0.1
q	Narcotine	11.50 ± 1.2	0.40 ± 0.1
s	Diazepam	12.11 ± 0.8	0.42 ± 0.3
e	Narceine	15.82 ± 1.1	0.55 ± 0.9
l	Flunitrazepam	28.67 ± 0.3	1
a	Caffeine	39.14 ± 0.2	1.36 ± 0.2
c	Pentobarbital	39.14 ± 0.2	1.36 ± 0.2
d	Barbital	39.14 ± 0.2	1.36 ± 0.2

Buffer: 50 m*M* phosphate (pH 2.35).
[a] See Fig. 2.

3.3. CZE2

The CZE analysis of basic drugs under alkaline conditions could, in principle, take advantage of closer differences between the buffer pH and the different pK_a values of the molecules, determining charge differences (in general negligible at acidic pH) which should increase the variability in charge-to-mass ratios of the analytes. Of course, weak bases will be non-ionized and therefore not susceptible to separation, but, on the other hand, acidic drugs will have a net charge and consequently could be separated. The choice of the running pH is therefore crucial, and should be optimized with respect to the specific compounds which are to be separated. In the present work, the pH of the CZE2 buffer (9.24) was on purpose the same as in the MECC experiments, in order to allow easier comparisons between the two techniques.

As expected, the migration sequence observed in our experiments (Fig. 3) was roughly related to the pK_a values of the drugs, with strong bases (e.g., amphetamine) migrating faster towards the detector than weak bases (e.g., cocaine); amphoteric narceine, morphine and MAM migrated after the electroosmotic flow (corresponding to

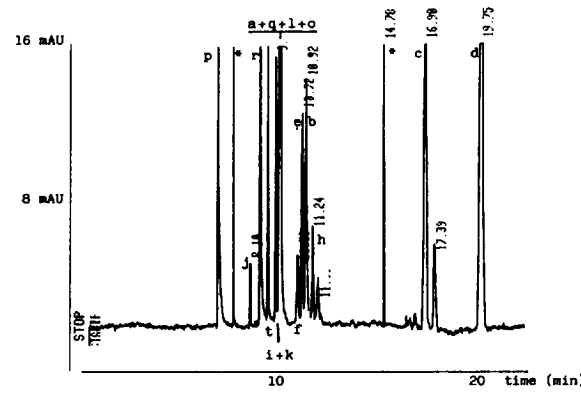

Fig. 3. Typical example of separation of the components of the drug test mixture according to the CZE2 method. Buffer: 50 m*M* borate (pH 9.25). For analytical conditions, see text. For peak identification, see Table 3.

caffeine), because of the negative ionization of the respective carboxylic or phenolic moieties. As for the acids, the two barbiturates have close pK values (7.8 for barbital and 7.9 for pentobarbital) and almost the same net negative charge; therefore, the difference in migration times (about 2 min) is believed to be dependent mainly on the difference in molecular mass (barbital M_r 184, pentobarbital M_r 248).

In Fig. 3, it is also evident that, when working with alkaline buffers, the huge electroosmotic flow "pushes" the analytes towards the detector, giving rise to crowding of most peaks in a narrow time frame.

Notwithstanding the heavy contribution of electroosmotic flow to the overall mobility of analytes, the intra-day R.S.D.s of absolute migration times ranged from 0.1 to 0.5% and the R.S.D.s of relative migration times from <0.1 to 0.5%, even with the manual instrument (Table 3).

The automated instrument was not tested at this pH because of limited availability.

The analytical sensitivity and efficiency of CZE2 was comparable to those of CZE1.

3.4. Correlation among MECC and CZE separation modes

The degree of mutual correlation between the three separation modes (MECC, CZE1 and CZE2) was investigated by comparing the respective migration order of the drugs (relative to flunitrazepam) by means of Spearman's test and principal component analysis.

As expected, CZE1 (running buffer pH 2.35) and CZE2 (running buffer pH 9.42), being based on a common, merely electrophoretic, separation principle, gave significantly correlated separations, with a Spearman's $r = 0.749$ ($p < 0.002$).

MECC and CZE2 were inversely and significantly correlated ($r = -0.865$, $p < 0.001$). It should be pointed out that caffeine, papaverine and narcotine were excluded because they were neutral at the pH of the CZE2 buffer and therefore not susceptible to electrophoretic separation. The inverse correlation suggests that the addition of anionic SDS micelles to the basic buffer affects the retention of the analytes not only on the basis of hydrophobicity, but also by ionic interactions with the charged groups of the

Table 3
CZE2 analysis of the test drug mixture

Peak	Compound	Migration time [min ± R.S.D. (%)]	Relative migration time ratio to flunitrazepam ± R.S.D. (%)
p	Amphetamine	4.94 ± 0.1	0.68 ± 0.1
j	Procaine	6.16 ± 0.1	0.85 ± 0.1
r	Cocaine	6.52 ± 0.5	0.90 ± 0.5
t	Tetracaine	9.81 ± 0.1	0.94 ± 0.2
i	Lidocaine	7.07 ± 0.1	0.98 ± 0.1
k	Heroin	7.07 ± 0.1	0.98 ± 0.1
a	Caffeine	7.19 ± 0.1	1.00 ± 0.1
l	Flunitrazepam	7.19 ± 0.1	1
q	Narcotine	7.19 ± 0.1	1.00 ± 0.1
o	Papaverine	7.19 ± 0.1	1.00 ± 0.1
f	MAM	7.96 ± 0.1	1.10 ± 0.0
e	Narceine	8.14 ± 0.1	1.13 ± 0.0
b	Morphine	8.28 ± 0.1	1.15 ± 0.0
h	Nalorphine	8.51 ± 0.1	1.20 ± 0.0
c	Pentobarbital	12.78 ± 0.1	1.77 ± 0.1
d	Barbital	14.88 ± 0.1	2.06 ± 0.1

Buffer: 50 mM borate (pH 9.24).
[a] See Fig. 3.

analytes. This can explain why amphetamine (pK_a 9.77) and cocaine (pK_a 8.7), still positively ionized at pH 9.24 and fast migrating in CZE2, migrated late in MECC. Barbiturates, late eluting in CZE2, were excluded from the micelles, because of their negative charge and therefore migrated relatively fast in MECC. These considerations tend to justify the inverse correlation between MECC and CZE2, which would be expected to be direct if the retention mechanism in MECC was based merely on hydrophobicity.

In the comparison between MECC and CZE1, we also intentionally omitted molecules not charged at the running buffer pH (i.e., caffeine, barbital and pentobarbital) for the above-mentioned reasons. The two methods were highly non-correlated in Spearman's test, with $r = -0.131$ ($p = 0.630$) for the basic drugs. The data resulting from Spearman's test were confirmed by principal component analysis, according which MECC and CZE1 are "orthogonal".

4. Conclusions

This work has confirmed the suitability of CE for the analysis of drugs of forensic interest, using MECC and/or CZE modes. Most of the test drugs were separated with the CZE1 and/or CZE2 methods, substantially according to mere electrophoretic principles. On the other hand, all of the 20 test substances were resolved with MECC, in which additional separation mechanisms based on the interaction with the anionic SDS micelles play an important role. The reproducibility of the migration times (particularly in MECC analysis) with the manual instrument used in this work was acceptable for practical application only if they were normalized on the basis of an internal standard (flunitrazepam) (in contrast, an automated instrument with control of capillary temperature, preliminarily tested in this study, proved highly reproducible also in terms of absolute migration times).

The principal aim of this work, however, was to investigate the degree of mutual correlation of the results from the CE methods used, to infer if the combination of two CE separation modes was effective in increasing the discrimination power and, hence, suitable for confirmation purposes.

The electropherograms of the components of the test mixtures were significantly correlated, as would be expected, for the two CZE methods (CZE1 and 2), notwithstanding a considerable difference in the running buffer pH values (9.24 for CZE2 and 2.35 for CZE1). Consequently, their combined use is believed to provide very little additional information. Surprisingly, CZE2 and MECC were also correlated in an inverse mode and with a high degree of statistical significance. This could be explained by postulating that the retention of basic drugs in MECC occurs not only as a function of the degree of hydrophobicity, but also based on ion-exchange interactions of the analytes with the external surface of the micelles. According to this assumption, strong bases, still bearing a strong positive charge at the pH (9.24) of the CZE2 and MECC buffers, will move fast in CZE, but will interact electrostatically with the anionic micelles, thus displaying a high "retention" in MECC. The opposite will occur with weakly basic drugs. Hence, according to this hypothesis, the electrostatic charge on the molecules affects both the migration velocity in CZE and the micelle-mediated retardation effects in MECC. Consequently, the two separation techniques, relying on a common physical/chemical characteristic of the molecules, could not be considered "independent" from each other and would not be suitable for mutual confirmation of results.

CZE1 and MECC, on the other hand, have proved to give highly non-correlated separation patterns in both Spearman's test and principal component analysis. As all the basic analytes have almost the same positive charge at the pH of the CZE1 running buffer, the electrophoretic mobility (based on the charge-to-mass ratio) depends on the molecular masses of the analytes, and not on charge differences (as in CZE2), which could affect also the MECC separation. This may explain the observed high non-correlation of the separation patterns and suggests the independence of the two separation techniques.

This is, of course, the basis for their complementary use for mutual confirmation.

This is particularly important if we consider that, according to the conclusions of the present work, two independent analytical techniques such as CZE and MECC can be carried out with the same instrumental hardware, by simply changing the running buffer and properly conditioning the capillary.

This highlights the great analytical versatility of CE, a characteristic which has often been overlooked, but which could be valuable in many forensic science laboratories, where the availability of different instrumentation is limited by resources, whereas the variety of analytical problems to be faced is probably the greatest in the analytical chemistry environment.

Acknowledgments

We gratefully thank Dr. David N. Heiger (Hewlett-Packard, Wilmington, DE, USA) kindly providing us with the HP 3D CE Model G1600A instrument. We also thank Dr. Luigi Perbellini (Department of Occupational Medicine, University Hospital of Verona), for carrying out statistical tests.

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