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Evaluation of capillary electrophoretic techniques towards systematic toxicological analysis

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Abstract

Two capillary electrophoresis (CE) methods were evaluated for their suitability in systematic toxicological analysis (STA). A test set of 25 barbiturates was analysed using capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC). Buffers used consisted of 90 m*M* borate set at pH 8.4 (CZE) and 20 m*M* phosphate, 50 m*M* sodium dodecyl sulphate set at pH 7.5 (MEKC). All analyses were carried out using fused silica capillaries using an electric field strength of 52.6 kV/m. The use of a reproducible identification parameter is very important in STA as it influences the identification power (IP). To deal with the poor reproducibility of the migration time, we introduced the corrected effective mobility. Inter-day reproducibilities of the latter parameter were $<0.6\%$ for CZE and $<0.5\%$ for MEKC, using daily prepared buffers. The IP of the methods was expressed by calculation of the discriminating power and the mean list length. Data obtained were compared to gas chromatographic and high-performance liquid chromatographic data, and correlations between all methods were calculated. It was shown that little correlation exists between chromatographic and electrophoretic techniques. The results indicated that CE has a good identification power for the application in STA, especially when a combination of methods having a low correlation is used. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

In many intoxications, if no specific suspicion of a defined poison exists, the analytical toxicologist needs to undertake a wide spectrum search to detect and identify ''any possible'' harmful compound. Since the number of toxicologically relevant substances is enormous, covering a wide and heterogeneous range of chemical compounds, a systematic approach is required. The logical chemical-analytical search for a potentially harmful substance whose presence is unsuspected and whose identity is unknown has been defined as systematic toxicological analysis (STA) [1,2].

In STA, powerful standardised analytical techniques are required, as well as databases containing analytical parameters of thousands of toxicologically relevant substances. The latter are required to try and match the analytical data of unknown compounds with the ones present in the database, thus providing the basis for identification. Also, toxicologically relevant substances which have been detected by screening procedures, such as immunoassays or receptor assays, need to be identified with more selective analytical methods. So far, several analytical techniques and systems have been evaluated with regard to their suitability for use in STA. Chromatographic techniques such as thin-layer chro-

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matography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been combined with appropriate detection modes, such as colour reactions, element-specific detections, UV–Vis/diode array detection, and mass spectrometry [3].

A modern separation technique with great potential is capillary electrophoresis (CE). CE is a flexible technique characterised by outstanding separation efficiency, high mass sensitivity, minimal use of samples and solvents, and fast analyses. These features make CE an attractive technique for toxicological analysis. Another important aspect of CE is its remarkable separation principle, which is different from those of more traditional analytical techniques such as chromatography. Since the separation principles of electrophoresis and chromatography are based on different physical-chemical properties of the analytes, the techniques can be considered non-correlated, which is advantageous for combining them in STA. Limitations of CE may be the low concentration sensitivity, when applied with UV detection, due to the short optical pathlength within the narrow capillary, and its vulnerability to matrix interferences. Therefore, the analyses of complex matrices, such as biological fluids, often require some kind of sample clean-up and preconcentration.

The two most common capillary electrophoretic modes are capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC). Apart from the electrokinetic interaction, the addition of micelles to the buffer in MEKC provides a second separation mechanism based on analyte–micelle interactions. CZE and MEKC are performed using the same equipment, and, therefore, it is possible to analyse a sample subsequently by both methods. The possibility to rapidly perform analyses by different separation mechanisms provides another important advantage of CE.

1.1. Enhancement of reproducibility

For STA applications, the use of a reproducible parameter for the discrimination between analytes (a so-called identification parameter) is of utmost importance [1,2]. For TLC, GC and HPLC, methods have been developed to correct the measured retention data. Thus, TLC data are based on corrected R_F values determined by the use of standards run alongside the unknown compounds on the plate [4–6], and retention in GC [7,8] and HPLC [9] is expressed in corrected retention indices (RI).

In CE, the identification parameter most often used is the absolute migration time t_m (i.e., the time required for the analyte to migrate from the point of injection to the point of detection). However, t_m is known to have a poor reproducibility in terms of relative standard deviations (RSDs), which makes it unsuitable to be used as an identification parameter. The major cause of non-reproducible migration times is the change in electroosmotic flow (EOF), which is the bulk flow of liquid due to the influence of the electric field on the layer of counterions adjacent to the negatively charged capillary wall. Unstable surface conditions of the fused silica capillary wall [10] or small variations in buffer pH [11] can cause small variations in the EOF. Furthermore, t_m may depend on a particular brand or batch of fused silica capillaries and on the instrumentation used, which can cause fluctuations in t_m between different laboratories. The choice of an identification parameter with a higher reproducibility than t_m is therefore highly important. An additional reason to use a parameter for substance identification other than t_m is the need for a standardised parameter. Since t_m depends on both migration distance and velocity, and therefore on capillary length and applied voltage, it is unsuitable as a reference parameter in databases used on an interlaboratory scale.

Various alternative identification parameters have been suggested in the literature to increase the reproducibility in CE. Sometimes the relative migration time, t_m^{rel}, is calculated against an internal standard by dividing t_m of the analyte by t_m of a standard which was added to the sample [12,13]. Yang et al. [11] calculated t_m and the total or apparent mobility (μ_{app}, see Eq. 2 below) of the analytes and divided these by t_m and μ_{app} of the EOF, respectively. The obtained measures were termed ''migration time ratio'' and ''mobility ratio'', respectively (denoted here as t_m^{ratio} and μ_{app}^{ratio}). The reproducibilities of these two measures were very similar, and higher than the reproducibility of t_m. Jumppanen and Riekkola [10] used two, three, or four marker compounds with known effective mobilities (μ_{eff}), and took changes of the EOF within

one run and the influence of factors such as viscosity into account. For MEKC analyses, the use of migration indices was introduced [14] to overcome the difficulty of assessing μ_{eff} of the marker compounds. It is evident that in MEKC, μ_{eff} is not only dependent on electrophoretic processes, but also on the partitioning of the analytes into the micelles. Therefore, the term ''overall mobility'' has been used to describe the mobility due to both processes in MEKC [15].

The use of μ_{eff} instead of t_m as an identification parameter has been reported [16–18] as an easy and effective way to improve the reproducibility. Since μ_{eff} is independent of the EOF and is in fact a property of the analytes, it is much more reproducible than t_m.

Obtaining μ_{eff} can be done using the following equation:

$$\mu_{eff} = \mu_{app} - \mu(EOF) \quad (1)$$

μ_{app} is directly related to t_m by:

$$\mu_{app} = \frac{l_d l_t}{t_m V} \quad (2)$$

in which l_d equals the capillary length to the detection window, l_t the total capillary length, and V the applied voltage.

μ(EOF) is calculated in a similar way using:

$$\mu(EOF) = \frac{l_d l_t}{t(EOF)V} \quad (3)$$

in which t(EOF) is the migration time of the EOF, measured using a neutral marker that moves at a velocity equal to the EOF. After obtaining μ_{app} and μ(EOF), μ_{eff} is readily calculated using Eq. 1.

The additional advantage of μ_{eff} is that it is a standardised parameter, since it is considered to be independent of capillary length and applied voltage. However, it is our experience that the reproducibility remains unaffected only when the voltage is adjusted while varying the capillary length, so that the electric field remains constant and consequently no temperature differences inside the capillary occur. The effect of a change in internal diameter cannot be easily corrected by a change in applied voltage.

In order to create a more reproducible and standardised identification parameter, we introduce here the ''corrected effective mobility'' (μ^c_{eff}). As mentioned before, this procedure we used has been successfully applied to paper chromatography and TLC analyses to correct for the non-reproducibility of R_F values [4–6]. To obtain μ^c_{eff}, we calculated μ_{eff} values and corrected these by interpolation between reference and measured values of μ_{eff} of standards. Reference values of μ_{eff}, denoted as μ^0_{eff}, were determined for each standard by analysing the standard mixture multiple times, and averaging the obtained μ_{eff} values. For the determination of the corrected effective mobility of analyte X, $\mu^c_{eff}(X)$, the standard mixture was analysed before and after each run of 10 samples. An equation of the graph of experimental and reference values for the standards was constructed and used to correct the experimental values of the analytes, $\mu_{eff}(X)$. According to this procedure $\mu^c_{eff}(X)$ can be calculated from:

$$\mu^c_{eff}(X) = a\mu_{eff}(X) + b \quad (4)$$

where a and b are constants derived from the reference values μ^0_{eff} of two standards, A and B, which are nearest to analyte X. If $\mu^0_{eff}(A)$ and $\mu^0_{eff}(B)$ are the reference values of the standards, and if $\mu_{eff}(A)$ and $\mu_{eff}(B)$ are the values for the standards measured in the mixture before or after analyte X, a and b can be calculated from:

$$a = \frac{\mu^0_{eff}(A) - \mu^0_{eff}(B)}{\mu_{eff}(A) - \mu_{eff}(B)} \quad (5)$$

$$b = \mu^0_{eff}(A) - a\mu_{eff}(A) \quad (6)$$

Substances A and B are chosen in such a way that A and B are the standards in the mixture with the values of $\mu_{eff}(A)$ and $\mu_{eff}(B)$ nearest to the value of analyte X, and so that $\mu_{eff}(B) < \mu_{eff}(X) < \mu_{eff}(A)$, where it must be noted that A elutes before X, which elutes before B.

1.2. Identification power

The suitability of a given analytical technique for STA can be evaluated by the calculation of several parameters which define the identification power (IP) of that technique for a given set of test compounds. The first IP parameter we will discuss here is the discriminating power (DP) [19]. The DP of an

identification method is defined as the probability that two substances selected at random from the test set would be discriminated by that method. The DP always lies between zero and one. The second IP parameter is the mean list length (MLL) [1]. A list length is defined as the number of feasible candidates found for a particular analytical value (such as an *RI* value in a chromatographic system). The average of all list lengths in a system gives the MLL, which lies between 1 and the number of substances in the test set. IPs have a value of 1 for ideal analytical methods, which can discriminate between each two substances in the test set. However, the two methods approach 1 from different directions: DP values range from 0 (bad IP) up to 1 (maximum IP), whereas MLL values will range from high values (bad IP) to 1 (maximum IP). DPs and MLLs can be calculated for individual analytical methods as well as for combinations of methods, including detection methods. It is important to note that DP and MLL values depend on the number and kind of compounds in the test set. A low number of test compounds will give an overestimation of the actual IP and will be less meaningful.

1.3. Objectives of the study

In this study, we selected 25 barbiturates as test substances (see Table 1) for the evaluation of CZE and MEKC suitability for STA. The analyses of barbiturates using CE has been described by a number of groups [20–24], but these studies focused on smaller numbers of analytes. We developed and compared a CZE and an MEKC method for the analyses of the test compounds, and calculated correlations between these methods and existing GC and reversed-phase (RP) HPLC methods. Furthermore, the reproducibility of the CE methods was studied using several identification parameters. Finally, the IP was determined for single methods and for combinations of methods, by the calculation of DP and MLL values. As can be seen from Table 1, barbiturates are structurally closely related and have similar pK_a values [25,26], which implies that they

Table 1
Structures of barbiturates used in the study and corresponding pK_a values ([25], except * [26])

Barbiturates	R_1	R_2	R_3	pK_a
Allobarbital	$-CH_2CH=CH_2$	$-CH_2CH=CH_2$	$-H$	7.680
Allylethylbarbituric acid	$-CH_2CH_3$	$-CH_2CH=CH_2$	$-H$	7.769
Allylphenylbarbituric acid	$-CH_2CH=CH_2$	$-C_6H_5$	$-H$	7.270
Amobarbital	$-CH_2CH_3$	$-C_2CH_2CH(CH_3)_2$	$-H$	7.865
Aprobarbital	$-CH(CH_3)_2$	$-CH_2CH=CH_2$	$-H$	7.904
Barbital	$-CH_2CH_3$	$-CH_2CH_3$	$-H$	7.859
Brallobarbital	$-CH_2CH=CH_2$	$-CH_2CBr=CH_2$	$-H$	7.464
Butalbital	$-CH_2CH(CH_3)_2$	$-CH_2CH=CH_2$	$-H$	7.703
Butobarbital	$-CH_2CH_3$	$-CH_2CH_2CH_2CH_3$	$-H$	7.863
Cyclobarbital	$-CH_2CH_3$	$-C_6H_9$	$-H$	7.513
Cyclopentobarbital	$-CH_2CH=CH_2$	$-C_5H_7$	$-H$	7.860
Heptobarbital	$-CH_3$	$-C_6H_5$	$-H$	7.633
Hexobarbital	$-CH_3$	$-C_6H_9$	$-CH_3$	8.194
Metharbital	$-CH_2CH_3$	$-CH_2CH_3$	$-CH_3$	8.297
Methohexital	$-CH_2CH=CH_2$	$-CH(CH_3)C\equiv CCH_2CH_3$	$-CH_3$	8.3*
Methylphenobarbital	$-CH_2CH_3$	$-C_6H_5$	$-CH_3$	7.8*
Pentobarbital	$-CH_2CH_3$	$-CH(CH_3)CH_2CH_2CH_3$	$-H$	8.032
Phenobarbital	$-CH_2CH_3$	$-C_6H_5$	$-H$	7.365
Probarbital	$-CH_2CH_3$	$-CH(CH_3)_2$	$-H$	8.048
Reposal	$-CH_2CH_3$	$-C_8H_{11}$	$-H$	Unknown
Secobarbital	$-CH_2CH=CH_2$	$-CH(CH_3)CH_2CH_2CH_3$	$-H$	7.869
Secbutobarbital	$-CH_2CH_3$	$-CH(CH_3)CH_2CH_3$	$-H$	8.012
Thiopental O→S	$-CH_2CH_3$	$-CH(CH_3)CH_2CH_2CH_3$	$-H$	7.6*
Vinbarbital	$-CH_2CH_3$	$-C(CH_3)=CHCH_2CH_3$	$-H$	7.499
Vinylbital	$-CH=CH_2$	$-CH(CH_3)CH_2CH_2CH_3$	$-H$	7.889

are an excellent test set to examine the IP of CZE and MEKC.

2. Experimental

2.1. Chemicals

Barbiturates were selected fom our in-laboratory collection of reference substances, obtained from commercial sources. Sodium dihydrogen phosphate monohydrate, boric acid, sodium dodecyl sulphate (SDS), methanol, and acetonitrile were purchased from Merck (Darmstadt, Germany). Formamide was purchased from Sigma (St. Louis, MO, USA). The water used was demineralised and further purified with an Elga ultra pure water system (Salm & Kipp, Breukelen, The Netherlands).

2.2. Equipment

Analyses were carried out on a Beckman P/ACE system 5500 capillary electropherograph (Beckman, Instruments, Fullerton, CA, USA) equipped with a diode array detector. Data were collected and interpreted using P/ACE System 5000 Series Software. Uncoated fused silica capillaries (Composite Metal Services, Hallow, UK) were used, of 57 cm (50 cm to the detector)×50 μm I.D..

2.3. Procedures

The running buffer used for CZE analyses consisted of 90 m*M* sodium borate, set at pH 8.4 using 0.5 *M* NaOH. The running buffer used for MEKC analyses consisted of 50 m*M* SDS in 20 m*M* Na_2HPO_4, set at pH 7.5 using 0.5 *M* NaOH. Buffers were freshly prepared on a daily basis. Buffer solutions were filtered prior to use through 0.45 μm RC 55 membrane filters (Schleicher & Schuell, Dassel, Germany).

Stock sample solutions were prepared by dissolving each barbiturate in MeOH, then diluting with water to a concentration of 1.25 mg/ml in approximately 25% MeOH. For analysis, samples were diluted to a concentration of 40 μg/ml with water and 0.01% of formamide was added as a neutral marker for EOF measurements. Samples were stored at 4°C.

The barbiturate standard mixture used to determine μ_{eff}^{c} consisted of barbital, phenobarbital, hexobarbital, secobarbital and methohexital. The mixture was chosen in such a way that the standards were evenly distributed over the analytical windows in both CZE and MEKC, and that they were of variable structure, thus representing the whole group of test substances. The standard mixture was analysed 94 times using MEKC and 35 times using CZE and the μ_{eff} values for the individual compounds were averaged. These averaged values were considered as μ_{eff}^{0}.

The following conditions were used for both CZE and MEKC measurements: Samples were injected hydrodynamically for 4 s (pressure 0.5 p.s.i.=35 mbar). A voltage of 30 kV was applied to the capillary (normal polarity), providing a current of ca. 20 μA and ca 60 μA, for CZE and MEKC, respectively. The diode array detector was set to monitor the effluent at 200 nm, and analyses were carried out at 25°C.

To reduce peak tailing, the outside surface of the capillary inlet was rinsed with water after sample injection. This was done by simply placing the capillary inlet into a vial filled with water for several seconds before it was placed into the buffer vial to carry out the separation. This significantly improved peak shape, as described earlier by Lux et al. [27], by preventing entry of the residual sample present on the outside surface of the capillary.

Between runs, the capillary was rinsed for 5 min with running buffer. After every five runs rinsing was done for 2 min with 0.1 *M* NaOH, 2 min with water, and 5 min with running buffer. To avoid buffer depletion due to electrolysis, the buffer in the vials was replaced after five runs.

3. Results and discussion

3.1. Method development

We investigated the use of buffers containing borate, phosphate and SDS at various concentrations and pH values, and the influence of several other factors affecting the migration behaviour of the

barbiturates such as the applied voltage, the capillary length, and the addition of organic modifiers. The final choice of separation conditions was a compromise between analysis time and resolution.

The buffers chosen on the basis of the obtained results consisted of 90 m*M* borate at pH 8.4 for CZE, and 50 m*M* SDS in 20 m*M* phosphate at pH 7.5 for MEKC separations. Higher buffer concentrations did not improve the resolution but generated higher currents, which is unfavourable since this can eventually lead to a reduced separation performance. The use of mixed buffers did not result in better separations than the use of single buffers. Upon raising the micelle concentration to 75 m*M*, analysis time increased, while the separation did not improve. By lowering the micelle concentration to 25 m*M*, analysis time decreased, however the resolution was reduced.

The influence of the pH was studied in the range from 7.0 to 9.0. The pH value of the buffers was found to be of major influence on the separation of the barbiturate mixture. A pH difference of 0.1 units resulted often in a different peak pattern. The pH value was therefore established to be a critical factor, which had to be strictly controlled (± 0.05 units).

An applied voltage of 30 kV resulted in faster separations and a better resolution compared to lower voltages (25 and 20 kV). Results obtained using capillaries of 47 cm and 57 cm total length (50 μm I.D.) were compared. A capillary of 57 cm total length provided a better resolution compared to a capillary of 47 cm total length, and resulted in only slightly longer migration times. Therefore, a capillary of 57 cm was chosen for further studies.

Also, the addition of two organic modifiers, i.e., methanol and acetonitrile, was studied. In CZE, the addition of 5 or 10% methanol or acetonitrile slightly improved the separation in terms of resolution, but increased the analysis time. In MEKC, the addition of 5% acetonitrile significantly decreased the analysis time but also the analytical window. Since the addition of an organic modifier resulted in an only slightly improved separation in CZE and a very small analytical window in MEKC, we decided not to use any modifier in subsequent studies. This approach allowed us to avoid evaporation of the volatile modifier which could influence the reproducibility of the analyses.

3.2. Comparison of CZE and MEKC

Under the chosen conditions, the negatively charged barbiturates are transported against the direction of their electrophoretic mobility towards the detector at the cathodic end of the capillary by the EOF. Fig. 1 shows the electropherograms of the mixture of the five standards as an example of the separations achieved. Total analysis time including rinse steps was usually in the order of no more than 10 min for CZE and 15 min for MEKC. High separation efficiencies ($>200\,000$ plates for most barbiturates) was achieved using both methods. With CZE, the resemblance of the chemical structures and the pK_a values of the barbiturates (see Table 1) resulted in a relatively small analytical window. The addition of micelles in MEKC clearly resulted in a different separation mechanism, reflected in various changes in elution order as compared to CZE. Also, MEKC provided a significantly increased analytical window resulting in an improved resolution. The migration of the analytes can be seen in Fig. 1 for the standard mixture and in Table 2 (CZE) and Table 3 (MEKC) for the other 20 barbiturates. It must be noted that it is difficult to compare the CZE and MEKC data, since different buffers and pH values were used.

The migration behaviour in CZE depends predominantly on the mass-to-charge ratio and thus on the pK_a value of the analytes. Analytes having a higher pK_a value (and thus a less negative charge) generally migrate faster towards the detector resulting in a lower (negative) effective mobility than those having a lower pK_a value. The migration behaviour in MEKC is different from CZE and almost independent of pK_a values. This is demonstrated by the correlations calculated between the effective mobilities and the pK_a values, which were 0.786 for CZE and -0.214 for MEKC, respectively. The influence of other factors, such as molecular size, interactions with the capillary, and the ability to form doubly charged species, apparently prevented a higher correlation between pK_a values and effective mobilities in CZE.

The migration behaviour in MEKC depends largely on the hydrophobic interaction of the analytes with the micelles. Hydrophobic components are more solubilised in the micelles resulting in a slower

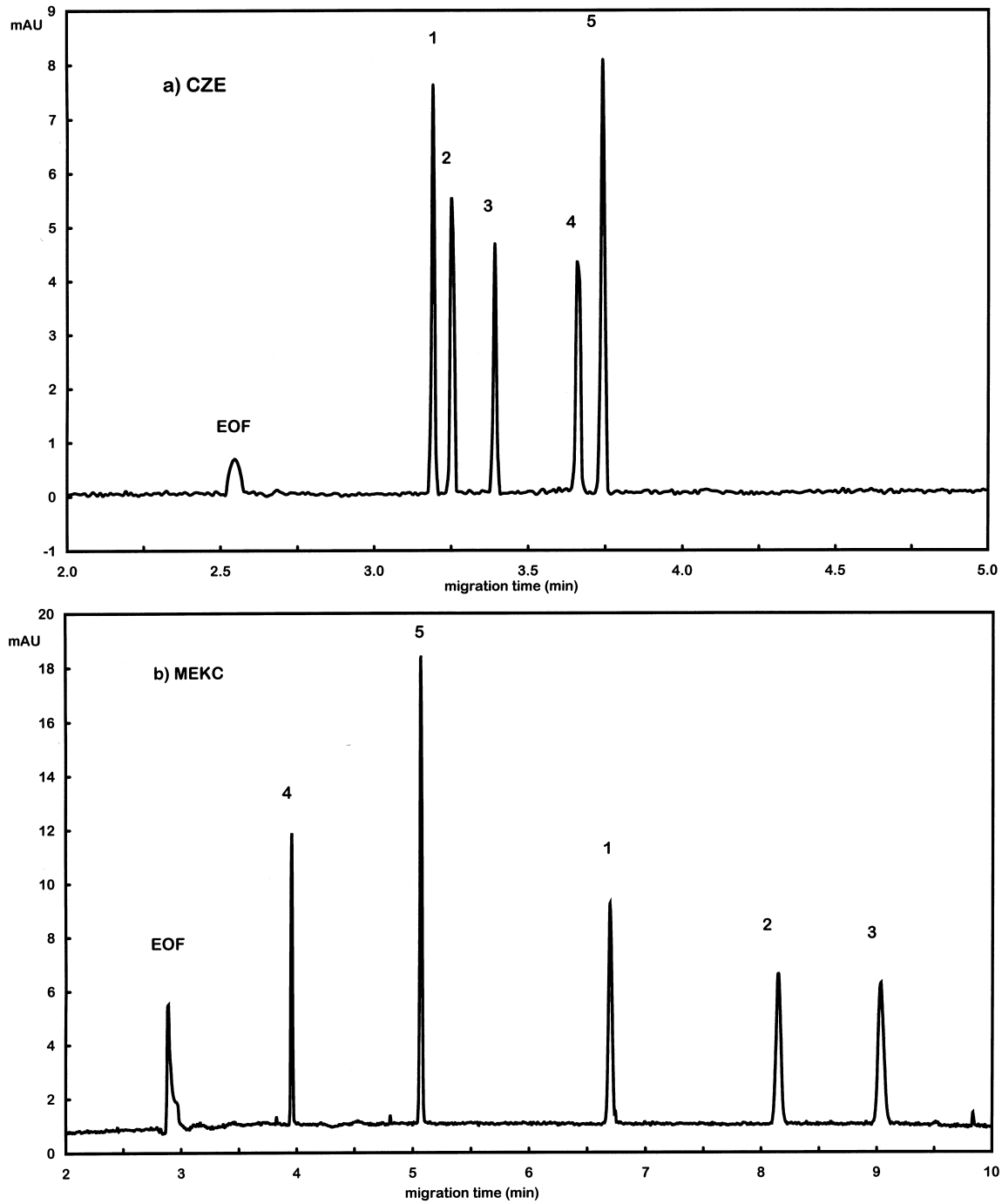

Fig. 1. Electropherograms of the separation of five barbiturate standards using CZE (A) and MEKC (B). 1 = Hexobarbital, 2 = methohexital, 3 = secobarbital, 4 = barbital and 5 = phenobarbital.

Table 2
Average values of five identification parameters and their RSDs (%) for the analysis of 20 barbiturates using CZE ($n=5$): t_m(migration time, min), t_m^{rel} (relative migration time to secobarbital), t_m^{ratio} (migration time ratio), μ_{eff} (effective mobility, $\cdot 10^{-8}$ m^2/V s), and μ_{eff}^c (corrected effective mobility, $\cdot 10^{-8}$ m^2/V s)

Barbiturate	t_m (min)	RSD (%)	t_m^{rel} value	RSD (%)	t_m^{ratio} value	RSD (%)	μ_{eff} (10^{-8} m^2/V s)	RSD (%)	μ_{eff}^c (10^{-8} m^2/V s)	RSD (%)
Allobarbital	4.13	7.8	1.08	1.2	1.51	3.0	−1.961	1.1	−1.962	0.8
Allylethylbarbituric acid	4.15	8.1	1.07	3.6	1.51	2.9	−1.955	0.8	−1.952	0.8
Allylphenylbarbituric acid	4.19	9.1	1.09	2.9	1.52	3.3	−1.965	4.2	−1.972	1.0
Amobarbital	3.81	6.7	1.00	1.5	1.40	2.2	−1.639	1.2	−1.639	0.3
Aprobarbital	3.96	7.6	1.03	1.7	1.44	2.7	−1.772	1.5	−1.771	0.6
Brallobarbital	4.22	4.0	1.09	6.2	1.52	1.6	−1.950	0.8	−1.941	0.5
Butalbital	4.00	6.7	1.04	2.0	1.45	2.3	−1.795	0.9	−1.794	0.9
Butobarbital	3.93	7.1	1.02	2.7	1.43	2.4	−1.723	1.1	−1.724	0.3
Cyclobarbital	4.00	6.7	1.04	2.2	1.45	2.3	−1.780	0.9	−1.778	0.8
Cyclopentobarbital	3.94	6.1	1.03	2.9	1.43	2.1	−1.742	1.1	−1.742	0.4
Heptobarbital	4.18	7.2	1.10	1.6	1.52	2.8	−1.984	1.0	−2.002	0.5
Metharbital	3.74	6.6	0.99	2.1	1.36	2.1	−1.503	1.6	−1.494	0.7
Methylphenobarbital	3.94	6.1	1.02	4.2	1.44	2.2	−1.768	1.2	−1.760	0.7
Pentobarbital	3.78	7.3	0.98	2.6	1.37	2.3	−1.560	1.4	−1.553	0.5
Probarbital	3.90	6.3	1.02	4.2	1.43	2.2	−1.735	1.1	−1.729	0.3
Reposal	3.94	4.1	1.04	3.7	1.42	1.5	−1.687	0.9	−1.683	0.8
Secbutobarbital	3.82	6.1	1.00	2.0	1.40	2.0	−1.645	1.2	−1.645	0.6
Thiopental	4.14	6.4	1.08	5.5	1.48	2.3	−1.850	1.1	−1.844	0.7
Vinbarbital	4.10	5.0	1.08	2.9	1.47	1.7	−1.803	0.8	−1.798	0.8
Vinylbital	4.02	5.1	1.05	5.7	1.43	1.7	−1.704	0.8	−1.700	0.7
RSD range		4.0–9.1		1.2–6.2		1.5–3.3		0.8–1.6		0.3–1.0
RSD average		6.5		3.1		2.3		1.1		0.6

migration as compared to less hydrophobic compounds. The logarithmic partition coefficient between *n*-octanol and water (log P_{oct}) is a measure used to express hydrophobicity, and may therefore be used to explain differences in the migration behaviour of components in MEKC compared to CZE. For example, phenobarbital, having a log P_{oct} value of 1.4 [26], migrated relatively fast in MEKC, but slowly in CZE. On the other hand, pentobarbital, with a higher log P_{oct} value of 1.9 [26], was solubilised into the micelles to a higher extent and migrated slowly in MEKC, but fast in CZE. Unfortunately, the log P_{oct} values for the majority of the analytes under study were not available, so that a more detailed evaluation of the relation between migrations and log P_{oct} values could not be performed. However, a high correlation between these parameters may not always exist since the mobility of the analytes in the aqueous phase also plays a role in their migration behaviour.

3.3. Correlations among CZE, MEKC, GC and RP-HPLC

Correlations were determined between μ_{eff}^c of the analytes in CZE and MEKC on the one hand, and between μ_{eff}^c of the CE methods and the *RI* values of previously validated GC and RP-HPLC methods [28] on the other. Fig. 2 shows correlation plots of each combination of two methods and the accompanying correlation coefficients for the 25 barbiturates.

As discussed previously, CZE and MEKC are based on different separation mechanisms, and these techniques are therefore considered to be non-correlated. Surprisingly, however, the CZE and MEKC data seemed to be somewhat inversely correlated ($r=-0.613$). This suggests that the addition of micelles to the buffer in MEKC not only introduces hydrophobic, but also ionic interactions between the negatively charged micelles and the analytes [12]. The inverse correlation may be explained by the

Table 3
Average values of five identification parameters and their RSDs (%) for the analysis of 20 barbiturates using MEKC ($n=5$, except * $n=4$): t_m (migration time, min), t_m^{rel} (relative migration time to hexobarbital), t_m^{ratio} (migration time ratio), μ_{eff} (effective mobility, $\cdot 10^{-8}$ $m^2/V\,s$), and μ_{eff}^c (corrected effective mobility, $\cdot 10^{-8}$ $m^2/V\,s$)

Barbiturate	t_m (min)	RSD (%)	t_m^{rel} value	RSD (%)	t_m^{ratio} value	RSD (%)	μ_{eff} (10^{-8} $m^2/V\,s$)	RSD (%)	μ_{eff}^c (10^{-8} $m^2/V\,s$)	RSD (%)
Allobarbital	4.87	11.8	0.68	6.0	1.59	4.5	−1.928	0.4	−1.951	0.6
Allylethylbarbituric acid	4.48	5.3	0.63	5.3	1.48	1.7	−1.696	0.3	−1.721	0.5
Allylphenylbarbituric acid*	5.90	7.5	0.83	6.1	1.95	3.8	−2.540	0.6	−2.564	0.2
Amobarbital	8.01	16.7	1.13	10.0	2.64	10.8	−3.205	0.9	−3.236	0.4
Aprobarbital	5.38	7.1	0.76	4.8	1.77	3.0	−2.275	0.5	−2.293	0.7
Brallobarbital	5.07	6.7	0.65	10.7	1.69	2.9	−2.169	0.5	−2.184	0.4
Butalbital*	6.10	9.8	0.81	6.1	2.00	5.2	−2.591	0.6	−2.612	0.4
Butobarbital*	6.30	10.2	0.84	4.6	2.06	4.3	−2.671	0.9	−2.692	0.4
Cyclobarbital*	6.09	9.5	0.81	3.5	2.00	5.0	−2.598	0.5	−2.618	0.3
Cyclopentobarbital*	6.73	10.3	0.90	3.0	2.21	5.9	−2.837	0.5	−2.861	0.4
Heptobarbital	5.11	5.0	0.65	8.5	1.68	1.9	−2.114	0.4	−2.131	0.4
Metharbital	4.85	4.9	0.62	9.1	1.61	1.6	−1.976	0.9	−1.995	0.9
Methylphenobarbital	7.46	9.2	0.95	5.9	2.46	6.2	−3.099	1.4	−3.119	1.3
Pentobarbital	8.77	9.6	1.12	4.4	2.87	6.4	−3.378	0.2	−3.406	0.2
Probarbital	4.87	5.0	0.62	9.0	1.61	1.8	−1.984	0.6	−2.003	0.6
Reposal	8.17	9.1	1.05	9.9	2.72	6.1	−3.334	0.5	−3.361	0.2
Secbutobarbital*	6.43	9.9	0.86	3.3	2.11	5.7	−2.727	1.1	−2.749	0.6
Thiopental	7.74	7.9	0.99	9.5	2.58	5.1	−3.234	0.6	−3.257	0.4
Vinbarbital	5.51	6.3	0.70	10.6	1.84	2.9	−2.407	0.2	−2.421	0.3
Vinylbital	8.16	9.5	1.04	9.3	2.71	6.2	−3.324	0.5	−3.353	0.2
RSD range		4.9–16.7		3.0–10.7		1.6–10.8		0.2–1.4		0.2–1.3
RSD average		8.6		6.8		4.5		0.5		0.5

following theory: Since the separations were performed at a pH near the pK_a values of the analytes, each barbiturate is partially ionised. When for a given barbiturate the anion/neutral ratio is high, the compound will migrate slowly to the cathode at the detector in CZE and appear relatively late in the CZE electropherogram. However, it will have little interactions with the micelle in MEKC and therefore appear early in the MEKC electropherogram. The opposite is true for analytes having a low anion/neutral ratio, which migrate faster in CZE and slower in MEKC.

MEKC and HPLC have a relatively high correlation ($r=0.854$) due to similarities in their separation mechanisms. CZE and GC are the two least correlated methods ($r=0.100$).

3.4. Reproducibility

An analytical method will only be valuable for STA if it provides reproducible as well as discriminating data. Here we discuss our findings on the inter-day reproducibility of our CE methods. We considered various parameters suggested for expressing data in CE and calculated their RSDs. The following identification parameters were used: t_m (as measured in the electropherogram), t_m^{rel} (relative to the standards secobarbital for CZE and hexobarbital for MEKC), t_m^{ratio} (relative to the EOF [11], which has a similar reproducibility as μ_{app}^{ratio}), μ_{eff}, and μ_{eff}^c. Table 4 shows μ_{eff}^0 values for CZE and MEKC, as well as the average μ (EOF) values of the EOF marker.

Tables 2 and 3 show the average values and RSDs of the identification parameters as obtained for CZE and MEKC, respectively. The average value of the RSD of t_m was 6.5% in CZE and 8.6% in MEKC. These high values can be explained by the facts that we prepared fresh buffers on a daily basis, which causes small fluctuations in buffer concentration and

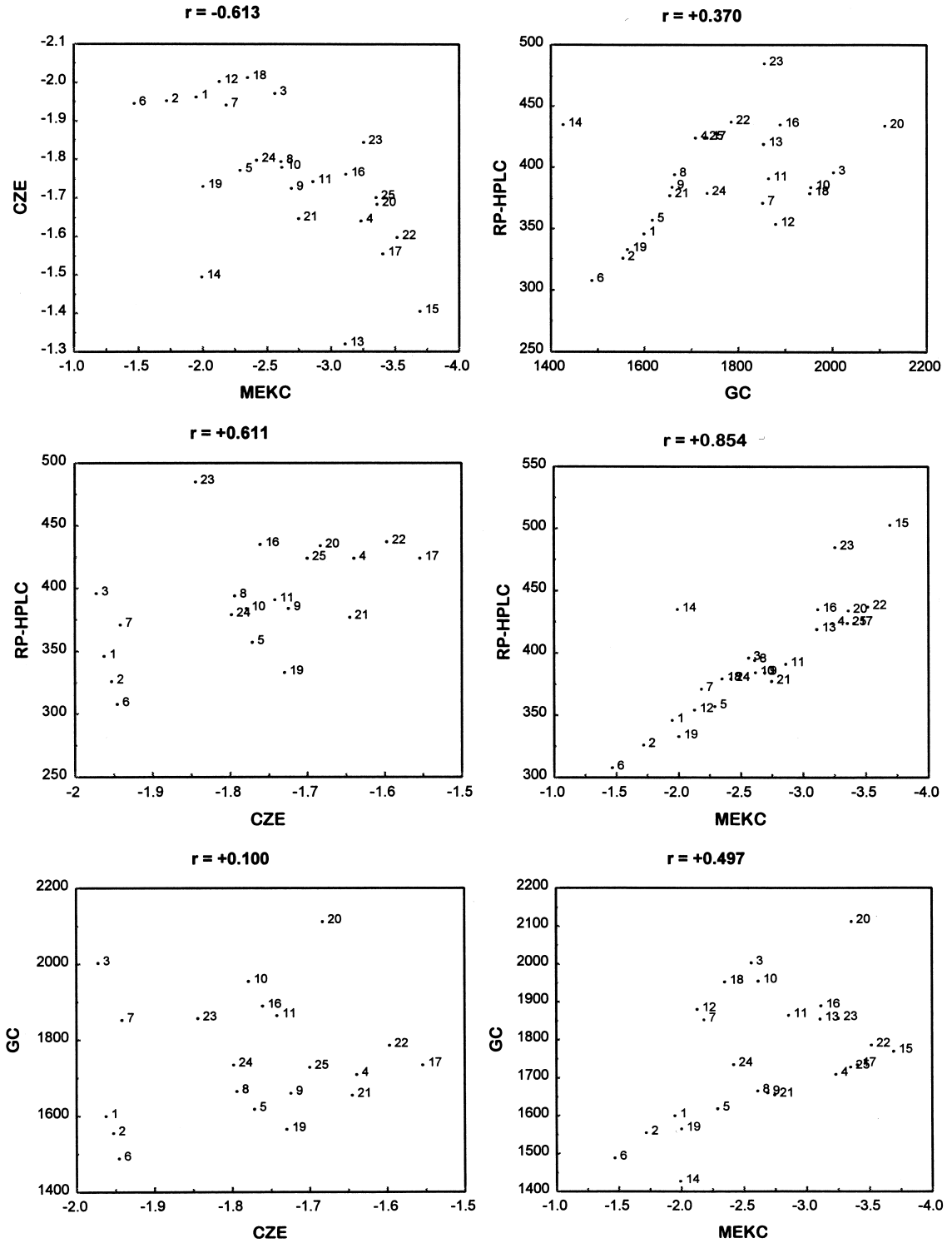

Fig. 2. Correlation plots and coefficients for each combination of two methods, including: CZE (μ_{eff}^{c}, $\cdot 10^{-8}$ $m^2/V\,s$), MEKC (idem), GC (*RI*), and RP-HPLC (idem). Note that μ_{eff}^{0} values are negative, and therefore CE axes are reversed.

Table 4
Reference values for μ_{eff}^{0} ($\cdot 10^{-8}$ $m^2/V\,s$) and RSD (%) of the barbiturate standards used in CZE ($n=35$) and MEKC ($n=94$), and the average μ_{eff} value ($\cdot 10^{-8}$ $m^2/V\,s$) and RSD (%) of the EOF marker in CZE ($n=99$) and MEKC ($n=94$)

	CZE		MEKC	
Barbiturate	μ_{eff}^{0} ($m^2/V\,s$)	RSD (%)	μ_{eff}^{0} ($m^2/V\,s$)	RSD (%)
Barbital	−1.945	0.4	−1.497	1.3
Hexobarbital	−1.319	1.7	−3.134	0.8
Methohexital	−1.404	0.8	−3.740	0.4
Phenobarbital	−2.013	1.3	−2.366	0.7
Secobarbital	−1.596	0.4	−3.551	0.5
EOF	5.760	4.0	5.238	4.0

pH, that the measurements were performed on different, non-successive days, and that we occasionally had to replace a blocked or broken capillary. From these factors, we suspect that pH fluctuations could be the most disturbing condition. For comparison, repeatabilities (i.e., intra-day RSDs) of t_m were $<1.0\%$ in CZE and $<2.0\%$ in MEKC. The RSD is higher in MEKC, which may be explained by the influence of the migration of the analyte–micelle complex on the migration of the analytes.

Fig. 3 shows the relationship between the RSDs of the five parameters and t_m in MEKC. This relationship was almost similar in CZE, but is not shown since the differences were smaller due to the smaller analytical window. From Fig. 3 it can be seen that the RSD of t_m increases with increasing t_m. Substances that remained in the capillary for a longer time were exposed longer to factors that influence the reproducibility, so that the RSD increased.

The results show that RSDs became smaller when any form of correction is applied. In general, the RSDs decrease in the order $t_m > t_m^{rel} > t_m^{ratio} > \mu_{eff} > \mu_{eff}^{c}$. The RSDs of t_m^{rel} are usually lower than the RSDs of t_m, but not always. This can be explained

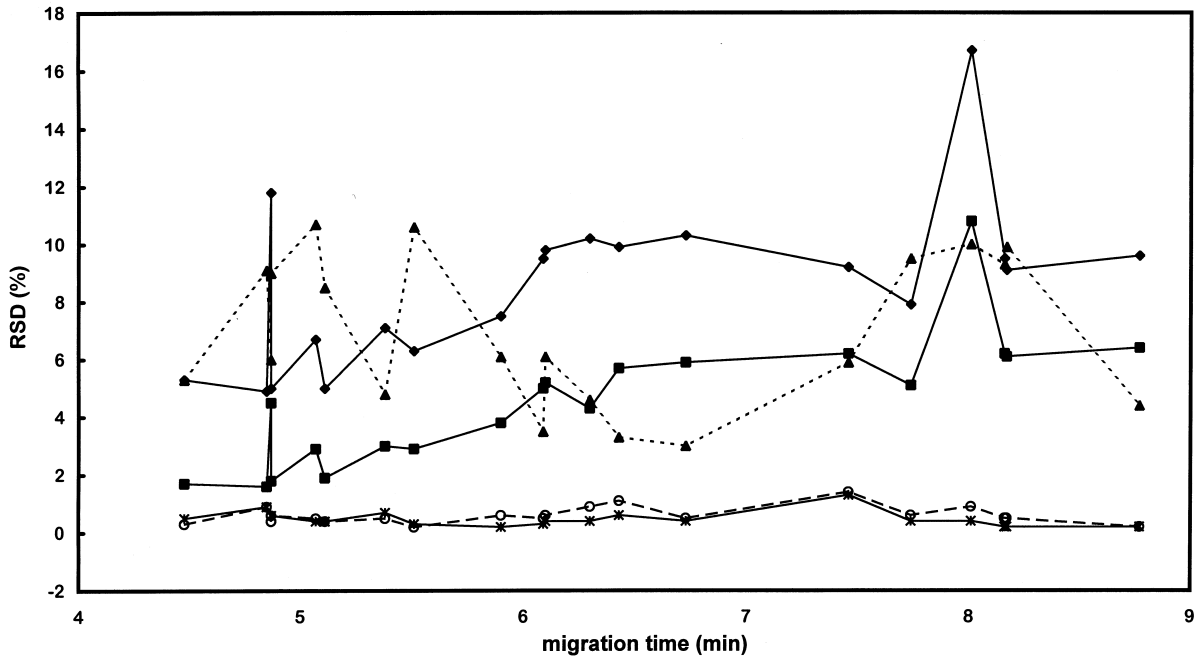

Fig. 3. Reproducibility of MEKC (RSD in%) against t_m (min) for five parameters: ♦, t_m; ■, t_m^{ratio}; ▲, t_m^{rel}; ○, μ_{eff}; ×, μ_{eff}^{c}.

by the fact that the bias in t_m of the standard also effects the RSD of t_m^{rel}, especially when the migration distance to the standard increases. The use of t_m^{ratio} results in lower RSDs than the use of t_m^{rel}. The RSD of t_m^{ratio} shows a downwards shift with regard to the RSDs of t_m. In contrast to the findings of Yang et al. [11], it does not seem to correct for outliers, nor does it reduce the upwards trend of the RSD with increasing t_m.

The most effective way to improve the reproducibility is the calculation of μ_{eff} or μ_{eff}^c. These parameters have the lowest RSDs of the parameters studied, with μ_{eff}^c being generally the lowest, with some exceptions in MEKC. In CZE, the RSDs of μ_{eff} and μ_{eff}^c are 1.1 and 0.6% respectively, and 0.5% for both parameters in MEKC. As compared with t_m, calculation of μ_{eff} and μ_{eff}^c results in an average improvement of 6- to 10-fold in CZE, and 17- to 24-fold in MEKC. Furthermore, both μ_{eff} and μ_{eff}^c reduce the upwards trend of the RSDs with increasing t_m, which was observed for the RSDs of t_m. Another important finding is that apparent outliers (such as amobarbital in MEKC) are corrected by the use of μ_{eff} and μ_{eff}^c. These results imply that the impact of the EOF, which is corrected for in μ_{eff} and μ_{eff}^c, is the cause of the upwards trend and the outliers of the RSD of t_m. Although t_m^{ratio} should correct for the irreproducible EOF in a different way, it does not reduce the upward trend with increasing t_m.

3.5. Discriminating power and mean list length values

A method to be applied in STA, should not only be reproducible, but also have a high IP. To express the IP of our methods, DP and MLL values were calculated for the individual CE methods and for the combination of CZE and MEKC. These values were compared to DPs and MLLs of standardised GC and RP-HPLC methods [28] for the analysis of the test compounds. The analytical parameters used for the calculations were μ_{eff}^c for CZE and MEKC, and *RI* for GC and HPLC. In all calculations, inter-day reproducibilities were taken into account, which need to be expressed as standard deviations (SDs) rather than RSDs.

To show the influence of the reproducibility on the IP of a method, calculations for CZE and MEKC were made on the basis of two different SDs. The first SD was 0.100, which was higher than 96% of the SDs in CZE and 88% of the SDs in MEKC; the second SD was 0.125, which was higher than all SDs measured in CZE and 96% of the SDs measured in MEKC.

For GC and HPLC, only interlaboratory SDs were available, which made comparison with our methods difficult. Tentative intralaboratory SDs were estimated by dividing the interlaboratory SD by 3. For calculations of the MLL, the desired probability of correctness can be chosen. MLLs were calculated for both 99% and 95% probability ($\alpha = 0.01$ and 0.05, respectively).

Table 5 shows that both DP and MLL are better for MEKC than for CZE, which can be explained by the larger analytical window in MEKC. Also, the values for MEKC are better than for GC and HPLC. However, neither the MEKC method alone nor any other single method is able to discriminate between all barbiturates in the test set. As expected, combining CZE and MEKC improves the IP, resulting in a DP of 0.990 and an MLL of 1.16 (for both α values). However, the combination of electrophoretic and chromatographic methods results in excellent IPs,

Table 5
MLL and DP values calculated for the analysis of 25 barbiturates using CZE, MEKC, GC, RP-HPLC, and the combination of each two methods[a]

Method(s)	SD	DP	MLL	
			$\alpha = 0.01$	$\alpha = 0.05$
CZE	0.100	0.823	4.32	3.40
	0.125	0.787	5.16	3.96
MEKC	0.100	0.973	1.44	1.40
	0.125	0.953	1.72	1.40
GC	5[b]	0.953	2.00	1.64
HPLC	3[b]	0.890	3.32	2.76
CZE/MEKC	0.125/0.125	0.990	1.16	1.16
GC/HPLC	5/3	0.987	1.16	1.16
CZE/GC	0.125/5	1.000	1.00	1.00
CZE/HPLC	0.125/3	0.973	1.20	1.00
MEKC/GC	0.125/5	0.993	1.00	1.00
MEKC/HPLC	0.125/3	0.990	1.12	1.00

[a] SD values are given for the methods used in the corresponding units, as well as the probability of correctness (α) for the calculation of MLL values.
[b] Estimated intralaboratory SD.

with an MLL ($\alpha = 0.05$) of 1.00 for all combinations. The combination of CZE and GC results in the best IP, which is 1.00 for the DP and MLLs for both α values.

Table 5 also shows the large impact of the reproducibility on IP. With a smaller SD, the IP of the method can improve substantially. However, the separation efficiency can also be important: Although GC yields a lower SD than MEKC, the DPs and MLLs are better in MEKC. This implies that MEKC is a better separation method for the identification of the barbiturates analysed than GC.

The combination of methods having a low inter-method correlation results in a higher IP. Since the separation mechanisms of the individual methods differ, the use of a combination of these methods will lead to an improved IP.

3.6. Concentration sensitivity

As mentioned earlier, one of the potential disadvantages of CE is the poor concentration sensitivity due to the short optical pathlength in the capillary. The lowest detectable concentration without preconcentration lies generally in the 0.2–5 mg/l range. In our experiments, we used analyte concentrations of 40 mg/l. Taking into account the obtained signal-to-noise ratios, we estimated that the limit of detection in our experiments is indeed in the 0.2–5 mg/l range. Toxic barbiturate serum levels (7–80 mg/l, depending on whether the barbiturate is short- or long-acting [29]) should therefore readily be detectable. However, since matrix compounds may interfere with the analysis, we expect that an extraction and preconcentration needs to be applied when analysing biofluids.

4. Conclusions

In STA, powerful analytical techniques are required for the identification of unknown compounds. Since single methods are unable to distinguish between all toxicologically relevant components, it is necessary to use a combination of methods which are based on different (separation) principles. Our findings indicate that CE has good potential for STA applications.

For the analysis of our test set of 25 barbiturates, little or no correlation existed between electrophoretic and chromatographic based techniques, which indicates that these methods can form effective combinations for STA applications. CZE and MEKC were shown to improve the IP when used in combination with chromatographic methods.

The use of a reproducible identification parameter is very important in STA as it influences the IP. The use of μ_{eff} and μ^{c}_{eff} instead of migration times in CE was shown to enhance the reproducibility, to reduce the upwards trend of RSDs with increasing migration time, and to correct for outliers.

It should be mentioned that for a complete evaluation of the CE methods interlaboratory SDs should be available, and we will try to obtain these in the future. Furthermore, the analysis of pharmaceuticals in biofluids will be investigated in future studies. The analysis of a larger number of substances, including different pharmacological classes, will be performed to support the general validity of our conclusions.

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