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Comparative use of three electrokinetic capillary methods for the determination of drugs in body fluids

Prospects for rapid determination of intoxications

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ABSTRACT

Three electrokinetic capillary methods, micellar electrokinetic capillary chromatography, capillary zone electrophoresis and capillary isotachophoresis, are shown to be well suited for the rapid screening and confirmation of drugs in serum and urine of patients with medical drug overdoses (intoxications), situations where rapid identification without precise quantification is needed. Patients' samples obtained from the emergency care unit were analysed in an instrument featuring on-column, fast forward-scanning multi-wavelength detection and the data were compared with those obtained by conventional methods. The drugs studied included salicylate, acetaminophen (paracetamol) and antiepileptics. In cases with high drug concentrations, body fluids can be injected directly or may have only to be diluted (urine) or ultrafiltered (serum) prior to analysis, providing results within about 30 min. Thus, electrokinetic capillary methods can be employed for rapid drug screening, provided that instrumentation with a database for peak identification is available.

INTRODUCTION

Emergency qualitative drug screening can provide valuable information to physicians faced with a confusing clinical presentation, atypical symptoms or signs and little or no history. For such cases, anticipated laboratory results may affect short-term management of a patient, this making drug screening an important part of a clinical service station [1]. Currently used analytical methods for drug screening are based on the principles of spectrophotometry, immunoassays and chromatography, with thin-layer chromatography [2] probably being most frequently employed. All of these techniques have advantages and disadvantages. The reagents for many of the immunological assays are available in kit form, together with highly automated instrumentation. This permits such analyses to be performed easily and rapidly. However, immunological techniques are prone to disturbances by molecules of similar structure (cross-reactivity). Many antibodies involved not only recognize the drug of interest, but also some of its metabolites. Moreover, these techniques are by nature unsuited to the simultaneous monitoring of several drugs and metabolites [3]. Chromatographic assays, on the other hand, provide specific results for multiple compounds but typically require extensive sample preparation and/or derivatization. Thus automation of chromatographic drug screening is complex [4].

For many years, analytical capillary isotachophoresis (CITP) performed in narrow-bore PTFE tubes of 200–500 μm I.D. was applied to drug monitoring [5]. Applications were developed in laboratories specializing in electro-

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phoretic techniques. However, partly owing to its complexity and the lack of automated instrumentation, CITP was never adopted as a routine drug assay methodology. Recently, instrumentation for electrokinetic separations in fused-silica capillaries of very small I.D. (25–75 μm) was developed [6–10] and the first papers reporting its use for drug monitoring in body fluids have appeared [11–23]. This type of instrumentation was shown to be suitable for high-resolution separations based on the principles of capillary zone electrophoresis (CZE), CITP and micellar electrokinetic capillary chromatography (MECC) [24,25].

For rapid analysis, minimum sample preparation is desirable. Sample preparation methods include simple liquid handling procedures (*e.g.*, centrifugation, dilution, filtration), release of the analyte from the biological matrix (*e.g.*, hydrolysis, sonication), the removal of endogenous compounds (*e.g.*, precipitation, ultrafiltration and extraction) and the enhancement of selectivity and sensitivity by analyte derivatization. In previous papers from our laboratory, the values of direct sample injection [18,19] and extraction [18–22] for drug screening by MECC was reported. In this work, MECC, CZE and CITP performed in the same instrument and with minimum sample pretreatment, including direct sample injection, dilution and ultrafiltration, were evaluated. Patients' samples obtained from the emergency care unit were analysed in an instrument featuring on-column, fast forward-scanning multi-wavelength detection and the data were compared with those obtained by conventional methods. The drugs studied include salicylate, acetaminophen (paracetamol) and antiepileptics. The data were employed to elucidate the pros and cons of the different electrokinetic capillary approaches for emergency analyses.

EXPERIMENTAL

Chemicals, origin of samples and routine methods of analysis

All chemicals were of analytical-reagent or research grade. The drugs employed as reference compounds were of European Pharmacopeia quality. Our own serum and urine were employed as blank matrices. Patients' samples were collected in our routine drug assay laboratory where they were received for drug screening. Salicylate, paracetamol, ethosuximide and primidone levels in serum were determined by automatic fluorescence polarization immunoassays (FPIA) on a TDx Analyzer (Abbott Labs., Irving, TX, USA). Phenobarbital was monitored by an automated enzyme immunoassay technique (EMIT) (Syva, Palo Alto, CA, USA) on a Cobas Fara centrifugal analyzer (Hoffmann-La Roche, Diagnostica, Basle, Switzerland). The immunoassays were performed according to the manufacturer's instructions using their reagent kits. Salicylate in urine was determined spectrophotometrically as the Fe(III) complex in acidic solution according to the method of Trinder [26], but without employing the precipitation reagent for proteins. Samples were stored at −20°C until further analysis.

Electrophoretic instrumentation and running conditions

The instrument with multi-wavelength detection employed in this work has been described previously [18–20]. Briefly, it featured a 75 μm I.D. fused-silica capillary of about 90 cm length (Product TSP/075/375; Polymicro Technologies, Phoenix, AZ, USA) together with a UVIS 206 PHD fast-scanning multi-wavelength detector with a No. 9550-0155 on-column capillary detector cell (both from Linear Instruments, Reno, NV, USA) towards the capillary end. The effective separation distance was 70 cm. Multi-wavelength data were read, evaluated and stored employing a Mandax AT 286 computer system and running the Model 206 detector software package version 2.0 (Linear Instruments) with windows 286 version 2.1 (Microsoft, Redmont, WA, USA). Throughout this work the Model 206 detector was employed in the high-speed polychrome mode by scanning from 195 to 320 nm at 5-nm intervals (26 wavelengths). With these settings the sampling rate was 3.69 data points per second and wavelength.

For all three methods, a constant voltage of 20 kV was applied and the cathode was on the detector side. Sample application was effected manually through dipping the anodic capillary

end into the sample vial and lifting it *ca.* 34 cm for a specified time interval. Conditioning between runs was achieved by rinsing the capillary with 0.1 *M* NaOH for 5 min and with buffer for 10 min. For MECC, a buffer composed of 75 m*M* sodium dodecyl sulphate (SDS), 6 m*M* $Na_2B_4O_7$ and 10 m*M* Na_2HPO_4 (pH ≈ 9.1) was employed. CZE analyses were executed with a 33 m*M* phosphate buffer of pH 8.3 (1.23 m*M* KH_2PO_4–32.1 m*M* Na_2HPO_4). CITP was performed with a leader of 10 m*M* HCl and histidine (pH 6.0) and a terminator composed of 10 m*M* 2-(N-morpholino)ethanesulphonic acid (MES) and histidine (pH 6.0). As described previously, the leader was placed in the anodic electrode vessel and the terminator was in the cathodic compartment and in the capillary [27]. All standard drug solutions were prepared in buffer or methanol at concentrations of 100–300 μg/ml. Spiking of blank and patients' samples was effected by addition of known aliquots of these standard solutions to the plain or pretreated body fluids.

Sample pretreatment for electrokinetic capillary analyses

Serum and urine samples were either injected as received or, prior to injection, centrifuged at 1500 *g* for 10 min and/or filtered using 0.2-μm Nalgene (25-mm diameter) disposable syringe filters (Nalge, Rochester, NY, USA). For the removal of the proteins, the samples were ultrafiltered at 1500 *g* for 20 min using the Centrifree Micropartition System (Grace, Amicon Division, Wallisellen, Switzerland).

RESULTS AND DISCUSSION

For three different cases, a comparative study of drug monitoring by MECC, CZE and CITP is presented. The first sample was serum of a patient with a suspected overdose of analgesics, the second a similar case for which both urine and serum were available and the third serum from a patient under multiple anticonvulsant pharmacotherapy.

Fig. 1 presents MECC data obtained for a patient's serum sample that was received for the

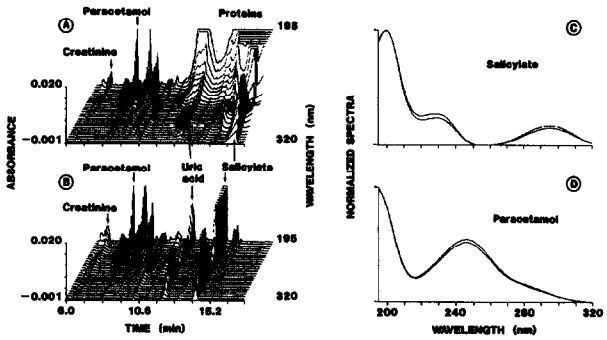

Fig. 1. Three-dimensional MECC data for (A) directly injected and (B) ultrafiltered patient's serum containing salicylate and paracetamol; (C) and (D) depict spectral identity proofs for salicylate and paracetamol, respectively. Sample injection time, 5 s; applied voltage, 20 kV; current, *ca.* 80 μA.

determination of salicylate and paracetamol. Using FPIA assays, the investigated serum was found to contain 3730 μ*M* salicylate and 160 μ*M* paracetamol, drug levels which are above those of the usual therapeutic ranges (salicylate 1100–2200 μ*M*, paracetamol 66–132 μ*M* [28]). With direct serum injection (Fig. 1A), the MECC data reveal a clear zone for paracetamol, and also peaks for the two endogenous marker substances, creatinine and uric acid. Also obtained is an indication of the presence of salicylate, which is shown to elute within unresolved matrix compounds, particularly proteins [19]. However, with ultrafiltration these interferences are removed and salicylate is also well monitored (Fig. 1B). For both drugs, excellent agreement between the extracted normalized absorbance spectra with those of a model run is obtained (Fig. 1C and D), revealing the unambiguous identification and purity of these zones. Thus, MECC of ultrafiltered serum has the potential to analyse rapidly for serum salicylate and paracetamol.

Comparing the electropherograms in Fig. 1A and B reveals interesting matrix effects. Ultrafiltration is shown to remove substantially interferences at elution times above about 12 min, permitting the formation of much improved zones of uric acid and salicylate. Second, the elution time intervals of all zones with serum injection are higher than those with the ultrafil-

trate. For example, paracetamol, uric acid and salicylate in Fig. 1A/B were detected after 8.59/8.33, 13.12/12.14 and 16.91/14.31 min, respectively. These data clearly emphasize the need for multi-wavelength detection for identification purposes. Retention (detection) times are not reliable parameters for zone assignment.

Omitting the solubilization of proteins (as with SDS in MECC) in an electrokinetic capillary system featuring untreated fused-silica capillaries makes direct injection of proteinaceous samples, particularly serum, difficult. Therefore, CZE and CITP analyses of ultrafiltered serum samples only were investigated. Analysing the patient's sample in Fig. 1 by CZE in a phosphate buffer of pH 8.3 gave the results presented in Fig. 2. Fig. 2A and B depict three-dimensional electropherograms of ultrafiltered serum blank and patient's serum, respectively. In this assay, paracetamol and salicylate are shown to produce clear peaks that elute in the same relative order with respect to uric acid as in MECC (Fig. 1) and to be clearly identifiable by normalized absorbance spectra (Fig. 2C and D).

Owing to the discontinuous buffer configuration, the data format in CITP is different to those obtained in MECC and CZE. Fig. 3 presents CITP electropherograms of (A) a blank and (B) an analysis of a model mixture of paracetamol and salicylate. Fig. 3A shows the complete three-dimensional UV data plot recorded with 5-nm increments for the wavelength

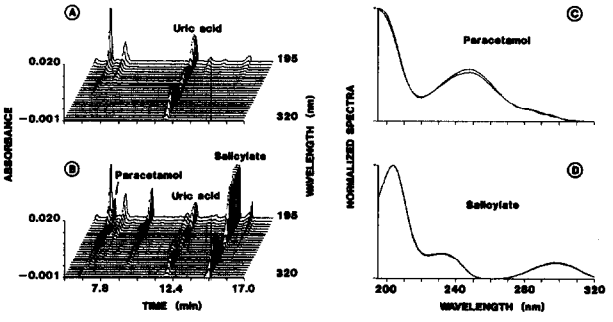

Fig. 2. Three-dimensional CZE data for (A) an ultrafiltered serum blank and (B) an ultrafiltered patient's serum (same as in Fig. 1B) together with the peak and reference spectra of (C) paracetamol and (D) salicylate. Sample injection time, 5 s. The applied voltage was a constant 20 kV and the current was observed to increase from about 70 to 78 μA during the experiment.

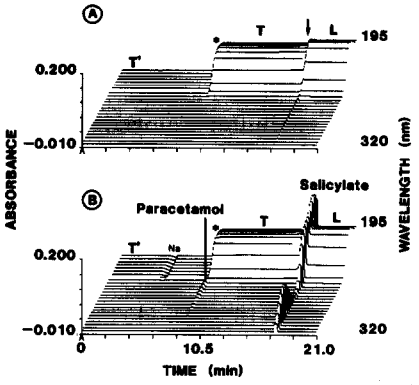

Fig. 3. Three-dimensional CITP data for (A) the blank and (B) a model sample consisting of salicylate (2.5 mM) and paracetamol (330 μM) having a sample injection time of 12 s. Leader (anolyte), adjusted terminator and initial terminator (catholyte) zones are marked L, T and T', respectively. The arrow and asterisk mark the leader–terminator boundary and the location of the initial leader–terminator interface (sample zone), respectively. The applied voltage was 20 kV. During the experiment a gradual current increase from 2 to about 6 μA was observed.

range 195–320 nm (absorbance *vs.* detection time *vs.* wavelength). Note that the total zone movement in this anionic CITP configuration proceeds in the direction opposite to that of electromigration [27,29]. The discontinuity at *ca.* 8.4 min (marked with an asterisk) represents the detection of the stationary boundary which is moved by the electroosmotic flow across the point of detection. The absorbance change observed originates from the change in the histidine concentration between the original (T') and the adjusted (T) terminator solutions. At the beginning of an experiment the UV absorbance was set to zero across the entire wavelength range. Finally, the leader–terminator interface (marked with an arrow) is monitored at *ca.* 16.8 min. The data presented in Fig. 3B reveal that salicylate is migrating isotachophoretically within the leader–terminator frame whereas paracetamol remains as an uncharged compound in the initial sample compartment and is therefore monitored within the T'–T transition. Sodium from the sample migrates zone electrophoretically and is detected through indirect absorption at low wavelengths at about 5 min.

CITP data obtained with (A) ultrafiltered

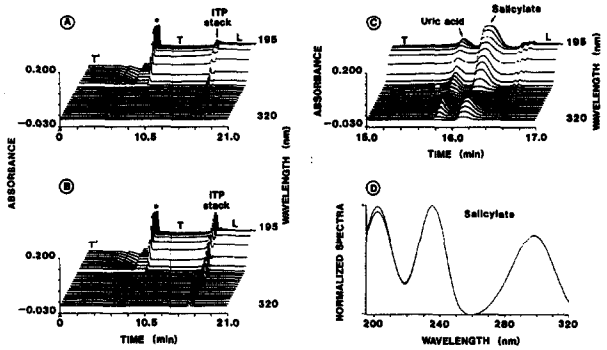

Fig. 4. Three-dimensional CITP data for (A) an ultrafiltered serum blank, (B) an ultrafiltered patient's serum (same as in Fig. 1B), (C) the ITP stack with an expanded time scale of (B) and (D) the peak and reference spectra of salicylate. The injection time was 6 s in all instances. Other conditions as in Fig. 3.

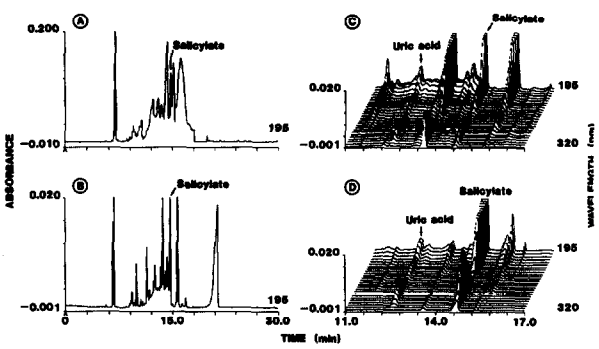

Fig. 5. Single-wavelength (195 nm) MECC data for (A) a directly injected patient's urine and (B) a tenfold diluted patient's urine, and multi-wavelength MECC data for (C) the tenfold diluted patient's urine (only part of the data with an expanded time axis are shown) and (D) an ultrafiltered serum from the same patient. Other conditions as in Fig. 1.

serum blank and (C and D) a patient's sample (same sample as used for Figs. 1B and 2B) are depicted in Fig. 4. Comparison of the electropherograms of the serum blank (Fig. 4A) with the buffer blank (Fig. 3A) shows that some endogenous compounds migrate isotachophoretically, forming a so-called ITP stack, whereas others remain between T′ and T or migrate zone electrophoretically within T′. Within the ITP stack, the substance with the characteristic absorption spectrum in the range 230–320 nm could be identified as uric acid. The ultrafiltered patient's serum provided an electropherogram (Fig. 4B) similar to that of serum blank (Fig. 4A). At first glance, it appears to be difficult to analyse salicylate in such a system. However, looking at the data depicted in Fig. 4C (which represent the ITP stack in Fig. 4B simply drawn on the expanded time scale) reveals the presence of salicylate. Identification of this zone was achieved by comparison of the background-subtracted, normalized absorbance spectrum with that in Fig. 3B. The good agreement between these spectra is depicted in Fig. 4D. Thus, CITP executed in fused-silica capillaries can easily be used to identify salicylate in ultrafiltered serum. Although paracetamol produces a small absorbance change in the 225–260 nm range at the T′–T interface (marked by an asterisk), its confirmation is impossible in that electrolyte system. No efforts were made to find a suitable electrolyte system for the CITP analysis of this drug.

MECC, CZE and CITP data obtained with a urine and a serum sample from a patient with suspected salicylate intoxication are presented in Figs. 5, 6 and 7, respectively. Using FPIA, the serum was found to contain 2998 μM of salicylate. The urine specimen tested markedly positive (>1 mM) for salicylate employing the modified spectrophotometric method of Trinder [26] (see above). MECC data for directly injected urine are depicted in Fig. 5A, for tenfold diluted urine in Fig. 5B and C and for ultrafiltered serum in Fig. 5D. In contrast to the analysis of the serum, direct injection of untreated urine is shown to

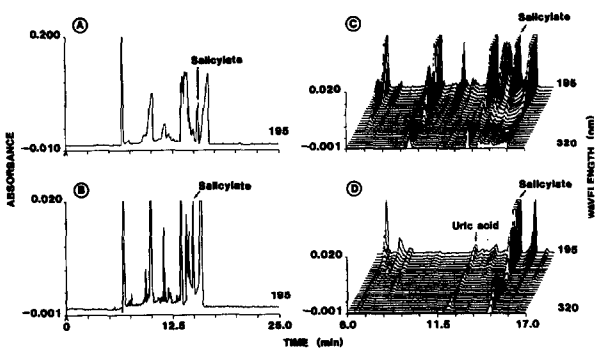

Fig. 6. Single-wavelength (195 nm) CZE data for (A) a directly injected patient's urine and (B) a tenfold diluted patient's urine, and multi-wavelength CZE data for (C) a tenfold diluted patient's urine and (D) an ultrafiltered serum from the same patient. Other conditions as in Fig. 2.

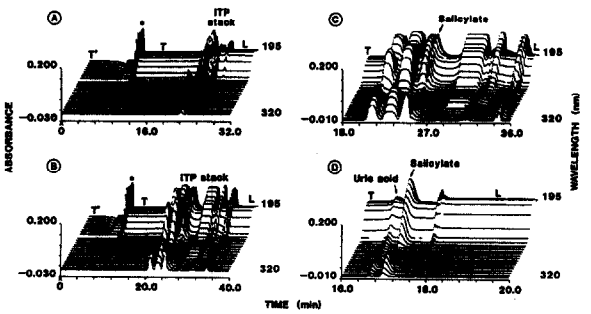

Fig. 7. CITP data for (A) a urine blank, (B) directly injected patient's urine (same as in Fig. 5), (C) ITP stack of the data of (B) with an expanded time scale and (D) ultrafiltered serum from the same patient. Injection time, 6 s. Other conditions as in Fig. 3.

overload the capillary column. Thus, for that example, sample dilution is required for the satisfactory determination of salicylate in urine. Comparison of the urine and serum data (Fig. 5C and D) reveals comparable salicylate concentrations in these two body fluids. However, the electropherogram obtained with urine is much more complex than that of ultrafiltered serum. The CZE analysis provided similar data (Fig. 6), which suggests that serum is the preferred body fluid for such an analysis. That the ionic matrix is more complex in urine than serum is further seen in the CITP data presented in Fig. 7. The ITP analysis of a urine blank (Fig. 7A) is shown to produce an ITP stack containing many more components than is obtained with a blank of ultrafiltered serum (Fig. 4A). The patient's urine injected as received provided the data depicted in Fig. 7B, its ITP stack with an expanded time scale being shown in Fig. 7C. This zone structure is about ten times longer than that obtained with the ultrafiltered serum depicted in Fig. 7D. Not surprisingly, identification of salicylate in the complex urine data proved to be more difficult than in serum. However, employing the comparison of normalized absorption spectra of eluting peaks (data not shown) provided the unambiguous presence of this drug in both fluids. It is important to realize that in CITP the detection time interval of a compound is strongly dependent on the sample matrix, this explaining the large difference in salicylate detection times seen in Fig. 7C and D. Hence the presence of salicylate in urine and serum was confirmed in the investigated patient's samples using three different electrokinetic capillary methods, MECC, CZE and CITP.

Fig. 8 represents the (A and B) CZE and (C and D) MECC analyses of antiepileptic drugs. Data for a model mixture consisting of four antiepileptic drugs and uric acid (Fig. 8A and C) and of an ultrafiltered serum sample from a patient under multiple antiepileptic drug pharmacotherapy (Fig. 8B and D) are compared. Using FPIA assays, the patient's serum was found to contain 209 μM of ethosuximide and 20 μM of primidone. A phenobarbital level of 105 μM was determined by an EMIT technique. CZE confirmation with the ultrafiltered serum was possible for ethosuximide and phenobarbital only; primidone in that system co-eluted with matrix compounds. However, all three anticonvulsants could be detected by MECC (Fig. 8D), even by direct injection of the serum (data not shown). The data show differences in the relative detection order of the compounds in the two methods. Primidone elutes in front of ethosuximide in CZE whereas the opposite occurs in MECC, and phenytoin is detected before and after uric acid in the CZE and MECC systems,

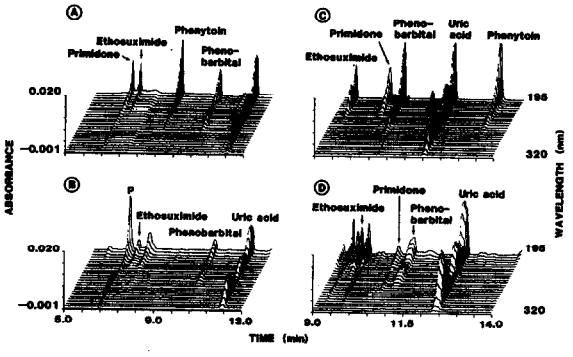

Fig. 8. Three-dimensional (A and B) CZE and (C and D) MECC data representing a model mixture of four antiepileptic drugs and uric acid [25 μg/ml each (A and C)] and an ultrafiltered serum from a patient under multiple anticonvulsant drug therapy (B and D). The conditions for the CZE and MECC runs are those in Figs. 2 and 1, respectively. P in (B) denotes the peak in which primidone is co-migrating with endogenous compounds.

respectively. Clearly, for anticonvulsants, MECC is preferred to CZE. This sample has not been subjected to CITP.

CONCLUSIONS

With its distinct advantages, including automation, small sample size, ease of buffer change, direct injection of body fluids and little sample preparation, compared with chromatographic approaches, modern electrokinetic capillary technology appears to be very attractive for the monitoring of intoxications. Three different methods were used in this work, MECC, CZE and CITP. The first two are easily applied whereas CITP requires very careful selection of buffer conditions and typically higher solute concentrations compared with MECC and CZE. Only with the use of the ITP spike technique, in which baseline-resolved UV absorption peaks of solutes are produced by bracketing the solute with discrete, non-absorbing spacers, CITP detection limits become comparable to those observed in MECC and CZE [30]. MECC is the most general approach because it permits the simultaneous determination of acidic, neutral and basic drugs. In cases with high drug concentrations, such as those reported here, body fluids can be injected directly or may have only to be diluted (urine) or ultrafiltered (serum) prior to analysis, providing results within about 30 min. However, in cases with drug concentrations at or below the μM (μg/ml) level, extraction with solute enrichment is required. This has been studied extensively for the MECC confirmation analysis of drugs in human urine [18–22]. Employing this approach, an analytical result is available within about 2 h. Thus, electrokinetic capillary methods can be employed for rapid drug screening, provided that instrumentation with a database for peak identification is available.

With absorption detection towards the capillary end, solute identification can be based on the retention (detection) behaviour and spectral analysis of eluting peaks over a range of wavelengths. In MECC and CZE, detection times are slightly dependent on the sample matrix, which calls for special care when using this parameter for solute identification. In CITP, zone identification by detection times is not possible. Multi-wavelength monitoring is an attractive approach for unambiguous zone assignment. Independent of the method used, the normalized spectrum of a peak is compared with a spectral computer library and segments of a peak can be compared with each other in order to assess peak purity. With all this information, a candidate list could be established followed by peak identification using a multi-parameter technique. If insufficient data or evidence for peak assignment are available, further evaluations and/or tests would have to be performed. Solute detection by other means, including mass spectrometry, could also be anticipated.

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