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Review

Determination of illicit and/or abused drugs and compounds of forensic interest in biosamples by capillary electrophoretic/electrokinetic methods

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Abstract

The application of capillary electrophoresis (CE) methods in forensic toxicology for the determination of illicit and/or misused drugs in biological samples is reviewed in the present paper. Sample pretreatments and direct injection modes used in CE for analysis of drugs in biological fluids are briefly described. Besides, applications of separation methods based on capillary zone electrophoresis or micellar electrokinetic chromatography with UV absorbance detection to (i) analysis of drugs of abuse, (ii) analysis of other drugs and toxicants of potential forensic interest and (iii) for metabolism studies are reviewed. Also, alternative CE methods are briefly discussed, including capillary isotachophoresis and separation on mixed polymer networks. High sensitivity detection methods used for forensic drug analysis in biological samples are then presented, particularly those based on laser induced fluorescence. A glimpse of the first examples of application of CE–mass spectrometry in forensic toxicology is finally given.

Keywords: Reviews; Drugs of abuse; Illicit drugs

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1. Introduction

Capillary electrophoresis (CE) is probably the most rapidly growing analytical technology that has appeared in the last two decades. Introduced by the fundamental papers of Mikkers et al. [1] and Jorgenson and Lukacs [2,3], CE has rapidly evolved as an independent technique, expanding beyond the application field of slab gel electrophoresis, traditionally limited to the separation of biopolymers, to include the analysis of inorganic anions and cations, metal chelates, pharmaceuticals and drugs (including enantiomers), hydrocarbons, organic acids, amines, carbohydrates, polymers and particles, fuels, dyes, explosives, etc., which traditionally were typical applications of chromatography.

A literature search for papers on ''capillary electrophoresis'' and its applications cited in the ''Analytical Abstracts'' database has enumerated more than 4000 citations and more than 2000 documents could be found on the Internet using the same keyword and ''Altavista'' search engine.

The great interest raised by CE is undoubtedly due to its high efficiency, mass sensitivity, minimum needs of solvent and sample volumes etc., but particularly to the high versatility in terms of separation modes, which, based on different physicochemical principles, display different selectivity. As it is well known, without changes in the instrumental hardware, CE separations can be carried out using capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE) and capillary isotachophoresis (CITP).

Probably, the main application field of CE is, at present, the determination of drug substances, and, indeed, the penetration of this technique into the pharmaceutical industry, after a slow start, is now a reality [4,5]. In the early 1990s CE, in the MECC mode, was introduced in the forensic field by the pioneering work of Weinberger and Lurie [6], who showed the potential of this technique for the separation of 18 illicit and/or controlled drugs, as well as heroin impurities and adulterants. Since then, relatively vast literature has been published in this field, with a particular focus on the analysis of drug exhibits [7], while less attention has been paid to the determination of illicit drugs and their metabolites in biological samples.

To the best of our knowledge, Wernly and Thormann first showed the feasibility of MECC analysis of drugs of abuse in human urine [8], and, probably, Thormann's group is still the most active in this area, even if several other researchers are now publishing actively.

Hence, the present review, based mainly on a bibliography search done in May 1997, cannot be fully updated, but is just intended to give an overview of the achievements, future possibilities and problems of the application of CE to the determination in biological samples of illicit, abused and controlled drugs and toxicants.

Due to the novelty of this analytical technique, particularly in the early papers, authors did not report fully validated methods, but just ''feasibility studies'' or examples to support their theoretical considerations; in other instances, the methods published were merely qualitative. However, we have often considered these papers worth mentioning, even if the methods were not formally ready for practical application, to help readers to start in the development of their own original methodologies.

For the same reason, a specific section on sample pretreatment has been included, as interferences from the different and numerous endogenous components often represent the major problem to be overcome in the application of instrumental analysis to biological samples.

2. Sample pretreatment and injection modes

Typically, biological samples subjected to drug analysis for forensic toxicology purposes are widely different and include almost any tissue and organ; however, a modern approach, in order to limit the workload due to the huge increase of casework occurring in recent decades, tends to limit as much as possible the specimens to be analyzed to those having a fundamental value for the purposes of the investigation.

In view of this, blood and urine are the first choice, the former for its importance in drug distribution to the organs and, often, for the good

correlation between drug concentration and the drug's acute toxic effects, the latter for the role of urine as a collector of drug metabolites for several hours after drug intake. Another biological tissue recently brought to the attention of forensic toxicologists is hair [9], in which many drugs penetrate at the root level, mainly from blood, during formation of hair matrix. In this tissue, organic compounds remain fairly unaltered for an extended time, because of lack of metabolism in the hair stalk and protection against degradation exerted by the hair matrix. Given an average hair growth rate of 1 cm/month, the analysis of a few centimeters of hair may give information on exposure of the subject to drugs (most often intake, but under some circumstances external contamination) during the corresponding months preceding hair collection.

In short, blood, urine and hair offer progressively increasing chronological windows for identifying drug intake, with correspondingly different diagnostic values.

For this reason, we will limit our attention to these tissues, which at present represent the majority of specimens analyzed for misused drugs and toxicants in most laboratories, but our considerations can easily be extended to other biological fluids (e.g., saliva, bile, vitreous humor) and solid tissues after, when needed, adequate homogenization and protein precipitation.

Although apparently similar, from an engineering point of view, to other instrumental analytical techniques (e.g., capillary gas chromatography and microbore high-performance liquid chromatography), CE shows such peculiarities in the separation mechanisms, that may affect the sample pretreatment procedures.

First, the injected volume is so small (a few nanoliters) that it is hardly handled by typical injectors used in liquid chromatography; moreover, the detector sensitivity is challenged by the minimal mass of analytes introduced in the capillary; optical detector sensitivity is further limited by the reduced pathlength and by the poor optical characteristics of the detection window, usually having a round section with a diameter of 20–100 μm. Because of these problems, when the analytes of interest are present in the biological samples at levels of a few μg/ml or below, sample concentration procedures may be required. On the other hand, the minimal need of sample makes possible the collection of specimens from the biological microcompartments ''just where analytes exert their activity'' (e.g., in single cells), where conceivably their local concentrations are high.

A second problem is represented by the need for sample desalting, if high efficiency is to be achieved, in order to avoid deleterious zone broadening (due to field drop) in the sample plug.

On the other hand, specific sample treatment and injection procedures may cause the sample to stack in the injection plug, thus allowing higher sample volumes to be loaded in the capillary, with consequent improvements in concentration sensitivity.

Finally, sample components may ''poison'' the capillary wall, affecting the shape of the injected plug and/or the electroendoosmotic flow (EOF) and, consequently, worsening separation efficiency and reproducibility.

Notwithstanding the above-mentioned problems, different approaches have been reported for the analysis of drugs in biofluids by CE, including direct injection of samples. An excellent review by Lloyd has recently appeared in Journal of Chromatography A [10] and the readers are referred to this publication for more detailed information. In the present section, we will briefly discuss this subject from a more specifically forensic toxicological point of view.

Most sample preparation techniques applied in CE for drug analysis are simply transferred from the wide body of experience accumulated in liquid (and gas) chromatography, but often this happens using schemes, procedures and devices (e.g., SPE disposable cartridges etc.) not specifically tailored for the microvolumes handled by the tiny capillaries, and consequently these techniques cause waste of sample, solvents, materials and unnecessary limitations in sensitivity. In reality, a specific strategy for sample preparation in CE has not yet been fully developed, but, recently, some miniaturized devices for on-line sample extraction, cleanup and analyte preconcentration have been introduced, at least as experimental prototypes [11].

In order to deal with sample matrix components, a common method in analytical biochemistry is sample dilution, as reported by Garcia and Shihabi [12], who proposed serum dilution in buffer with ionic strength

10–20 times lower than the electrophoresis buffer for the CZE determination of theophylline. The separation buffer, consisting of 100–300 m*M* borate pH 8.8, had a fairly high ionic strength. In addition to the minimization of matrix interferences, the dilution of serum proteins and ions in combination with the high molarity of the running buffer exerted a sample stacking effect, which enabled a larger volume of sample to be introduced, without excessive peak broadening. However, this approach is, practically, of limited value for drug analysis, because sample dilution is deleterious to the concentration sensitivity of the CE methods, which is intrinsically limited also by other instrumental factors (short optical pathlength, minimal injected volumes etc.). Thus, only drugs active at high concentrations, like theophylline, barbiturates etc. may be assayed using sample dilution methods.

Although it usually contains a higher concentration of drugs and metabolites, urine is extremely rich in interfering components and, consequently, the resolving power of CE is often not sufficient to adequately separate the analytes of interest. However, urine dilution in water (3:10, v/v) prior to CZE could be used successfully for the determination of ephedrine and norephedrine [13].

Despite the abundance of endogenous (and possibly exogenous), potentially interfering compounds and the high salt and protein concentration in plasma, serum and urine samples, CE has proved to be able to deal with direct injection, after simple filtration/centrifugation to remove particulate material which could clog the capillary.

However, it is worth pointing out that under these ''stressing'' analytical conditions:

- protein-bound drugs and conjugated metabolites migrate differently from the ''free'' standards which are currently available;
- the sensitivity of the system may be limited by the inherent impossibility of sample preconcentration and by the high conductivity of the biological specimens (unless specific procedures are carried out to avoid excessive peak broadening);
- almost all the selectivity of the analysis relies on electrophoretic separation, because detection selectivity is also limited by the need of working at a nonselective wavelength of 200 nm to achieve the required sensitivity with UV absorbance detectors.

Thus, it can be easily understood that these limitations severely affect the usefulness of the direct injection method for analytical toxicology purposes.

Both CZE and MECC have been used for ''direct injection'' analyses of drugs of potential forensic interest. CZE, in particular, requires the use of high ionic strength running buffers to overcome the disturbing effects of salts naturally present in the matrix. If these conditions can be met, relatively low detection limits are obtained, as shown by Li et al. [14], who reported a sensitivity of 80 ng/ml for dextromethorphan and its metabolite dextrorphan in enzymatically deconjugated urine. Serum, because of the higher protein content, is less suitable for direct injection analysis. Although the use of high concentration running buffers ($\geq$100 m*M*) and vigorous washings with acid/alkali or SDS solutions (see Ref. [10] for an overview) appear effective in maintaining an acceptable efficiency and reproducibility, the concomitant problems of analytical sensitivity and selectivity have so far hindered the adoption of direct injection procedures in drug analysis for forensic purposes.

MECC offers additional chances to tune selectivity due to the presence of a micellar pseudostationary phase in the system, which mimics a reversed-phase liquid chromatographic stationary phase. Consequently, MECC appears more suitable for direct injection of biological samples than CZE, due also to the denaturing effect of SDS, the most common micellar agent, on proteins. These biopolymers, in the presence of SDS, become highly negatively charged and, bearing the same sign as the fused silica capillary, are prevented from interacting with the walls. Caslavska et al. [15] have reported successful use of MECC with direct injection of urine for the determination of caffeine, mephenytoin, dextromethorphan and the respective metabolites for the identification of metabolic phenotypes. In order to get a more reliable identification of the peaks and to help to resolve analytes from the many urine interferants, UV absorbance detection was carried out with a multiple wavelength fast scanning detector, thus combining migration and spectral characteristics of the peaks. The advantages of MECC over CZE in dealing with high protein concentration in the sam-

ples are more evident with serum and plasma samples. Phenobarbital, ethosuximide and primidone could be detected by direct serum injection [16]. Also, saliva could be injected directly for the MECC analysis of antipyrine [17]. Moreover, the denaturing effect of SDS was reported to cause the release of protein-bound drugs, even in cases of high binding percentages, as for naproxen [18], allowing the direct analysis of ''total'' drug content in serum or plasma.

When required for determination of the ''free fraction'' of a drug, ultrafiltration has been proposed to remove serum proteins with the bound fraction of drugs, under nondenaturing conditions, prior to sample injection in MECC [19].

As reported by Zhang and Thormann [20], single-level external calibration can be used to produce reliable quantitative determinations of drugs by MECC with direct injection of serum. According to the authors, calculation on the basis of peak height provides results with higher precision and accuracy than those based on peak areas. Eventually, to achieve the best accuracy, calibrators must be dissolved in the same matrix as the unknown samples.

Notwithstanding several attempts to avoid any sample preparation, as discussed above, the great majority of authors apply procedures to simplify the complexity of biological matrices and, possibly, to enrich the extract in analytes of interest.

One of the simplest and most traditional sample preparation methods in analytical biochemistry, which was first transferred to CE, is protein precipitation, followed by centrifugation and injection of the clear supernatant. In drug analysis, this strategy may be useful not only for getting rid of analytical interferences caused by these major components in any biosample, but also, if necessary, for releasing drugs from transport proteins, which may bind more than 90% of drugs.

The need to maintain a low sample conductivity in CE, to hinder zone broadening, has precluded sample precipitation with salts or acids, while addition of organic solvents was the method of choice. Indeed, organic solvents like alcohols and acetonitrile, added in different proportions to the sample (from 1:1 to 4:1 or more), show a good denaturing effect, reduce the conductivity of the resulting mixture and may be easily removed by evaporation, to counteract the sample dilution inherent in their use. Acetonitrile was reported as the best solvent for serum deproteinization (in a proportion acetonitrile–serum of 3:2) prior to CZE by Shihabi [21] for the analysis of drugs (theophylline, phenytoin, phenobarbital), particularly with electrokinetic injection.

In effect, CZE analysis showed increased efficiency and sensitivity, which were attributed to stacking effects determined by the high resistivity of the water–acetonitrile mixture in which sample components were dissolved, in comparison to that of the separation buffer. Conversely, in our experience, acetonitrile, because of its strong denaturing effect on serum proteins, may cause analyte loss due to coprecipitation. Hence milder denaturing agents (e.g., methanol or ethanol) may be preferable [22,23].

Additional advantages of protein precipitation are the release of bound drug fractions and solubilization of analytes poorly soluble in aqueous buffers, but a notable disadvantage is sample dilution. The influence of acetonitrile deproteinization on the reproducibility of CZE separation was studied by Johansson et al. [24], who concluded that, to obtain reproducible results, standards and samples should be prepared in the same solvent. Besides, high percentages of organic solvents in the injection mixture may negatively affect MECC separations, because of interference with the partition equilibria of analytes with the micelles at the starting end of the capillary [25]. To overcome these problems, solvents can be easily removed by evaporation [26,23] or extraction [27] before injection. However, due to the persisting presence of interfering compounds, notwithstanding attempts to preconcentrate the extract [26] or to inject under stacking conditions [24], sensitivity levels below 1 μg/ml, e.g. Ref. [26], are rarely reported with CE–UV methods, after serum/plasma protein precipitation.

Conversely, the above approaches are rapid, simple and low-cost and are therefore particularly suitable for clinical chemistry applications.

From a forensic toxicology point of view, however, more selective sample pretreatments are generally preferred, which can contribute to the overall selectivity of the analytical methodology and, by lowering the matrix related noise, can improve sensitivity.

Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) methods have been used as sample pretreatments for the CE analysis of drugs of foren-

sic interest. Both are based on partitioning of the analytes between the sample and another phase, in liquid form or bound onto a solid substrate. The fine tuning of the extraction methods allows excellent removal of proteins, small ions and many other endogenous compounds with excellent recoveries of analytes. In general, LLE extraction methods have a broad spectrum of extractable drugs [28]. On the contrary, SPE methods are more selective and, consequently, give cleaner extracts, but a more limited number of analytes can be extracted simultaneously with a given method. In both cases, the extracted mixtures can be easily dried off and reconstituted with a small volume of suitable solvent, thus achieving concentration factors up to 100 times.

The usual SPE extraction mechanism for drugs is reversed-phase on C_{18}-silica or polymeric packings, but recently double-mechanism stationary phases have been introduced with increasing success in analytical toxicology. Both C_{18}-silica [29] and double mechanism (reversed-phase and cation-exchange) cartridges [30] proved excellent for CE analysis of therapeutic and/or abused drugs. The latter, being particularly selective towards matrix components which can ''poison'' the capillary, spoil the formation of the injection plug and hinder detection, allowed up to 50-fold sample concentration before injection, achieving detection limits of about 100 ng/ml for drugs of abuse.

As already mentioned, CE, particularly in the CZE mode, offers peculiar methods for sample enrichment during injection, known as sample stacking. The most widely used techniques are based on conductivity differences between the sample and the electrophoretic background electrolyte: the lower the salt concentration in the sample, the higher the analyte concentration that can be achieved. Another approach is based on the application of isotachophoretic preconcentration steps, which can be performed in the same column where separation is then carried out or in a precapillary, from which the analyte zones are transferred to the separation capillary.

A large number of papers have been devoted to the study of these techniques, most of which are beyond the scope of the present review; the readers can find more information in a short review by Schwer [31]. Moreover, Zhang and Thormann showed an excellent application of head-column field-amplified sample stacking to amiodarone and desethylamiodarone determination in two recent papers [32]. The high content of organic solvent and the presence of small amounts of protons (50–100 μM) in the sample, resulting in a low conductivity medium, allowed up to 1000-fold sensitivity enhancement, still with acceptable reproducibility, supported by R.S.D. values around 3–6% for inter- and intra-day determinations with the use of internal standardization. The impressive limit of detection of <0.681 ng/ml in serum with UV absorbance detection, after liquid–liquid extraction of 2–20 μl of serum, was reported in [33].

A combined cleanup and concentration approach integrating a particular LLE form, using minimal amounts of organic liquid (i.e. supported liquid membranes), and a double stacking technique allowed bamuterol (a basic drug) detection by CE–UV absorbance in plasma in the low nM range [34].

A further possibility of enhancement of concentration limits of detection in CE is offered by the newly introduced preconcentration on polymeric styrene–divinyl benzene copolymer membranes, placed into the capillary system, which has been applied particularly in CE coupled to mass spectrometry [35].

On the basis of the above considerations and of the recent improvements in instrumental sensitivity (e.g., less noisy UV detectors, Z shaped cells, laser fluorescence and electrochemical detectors, mass spectrometers etc.), we believe that by combining careful sample preparation and suitable sample stacking procedures, in the near future, it will be possible to overcome all the still existing sensitivity limitations of CE, which have hindered so far the application of this technique to the analysis of biological fluids for many toxicologically relevant drugs.

3. Separation by CZE–MECC with UV absorbance detection

3.1. Analysis of drugs of abuse

Almost all the existing commercial CE hardware in its ''standard configuration'' features automated injection in both electrokinetic and hydrodynamic

modes, thermostated separation under constant voltage or constant current conditions, and ''in capillary'' UV absorbance detection, by means of filter or monochromator single wavelength detectors or with more sophisticated fast scanning or diode array multiwavelength spectrophotometers.

This has oriented applications towards methods based on UV absorbance detection, which although characterized by a broad spectrum of applications, in CE show specific limitations in sensitivity and selectivity, due to the limited choice of wavelengths at which most of the compounds display sufficient molar absorptivity (i.e. around 200 nm) to allow sensitive detection.

For this reason, most of the overall selectivity required by the method is based on that offered by the electrophoretic/electrokinetic separation. Fortunately, CE is intrinsically a high efficiency technique able to produce hundreds of thousands of theoretical plates in a few minutes and shows a good peak capacity, with a resolving power much higher than any other separation technique in the liquid phase. Thus, even simple CZE methods, in which the separation mode is based on plain electrophoresis, have been successfully applied to the analysis of therapeutic and illicit drugs in body fluids. However, because of a more sophisticated separation mechanism including pseudochromatographic retention phenomena (mimicking reversed-phase chromatography), which allows also the determination of nonionized compounds, MECC has received the greatest attention in drug analysis. A review on this subject encompassing as many as 243 references has recently been published by Nishi and Terabe [36], and readers are referred to this publication for more information on fundamentals, optimization, instrumentation, validation and for a general overview of applications in drug purity testing, assay of drugs including hydrophobic drugs, amino acids, vitamins, peptides, natural products, enantiomers and physicochemical properties of drugs such as distribution coefficient and thermodynamic quantities in micellar solubilization as well as *n*-octanol–water partition coefficient.

To the best of our knowledge, Weinberger and Lurie [6], in 1991, first applied CE, in the MECC mode, to the analysis of illicit/abused drug substances. The authors used 50-μm I.D. bare silica capillaries, 25–100 cm in length, and a background buffer consisting of 85 m*M* SDS, 8.5 m*M* phosphate, 8.5 m*M* borate, at a pH of 8.5, containing 15% of acetonitrile. The applied voltages were 25–30 kV; detection was by UV absorption at 210 nm. Under the described conditions, it was possible to separate, with high efficiency, a mixture of as many as 18 drugs, including psilocybin, amphetamines, benzodiazepines, PCP, cannabinoids etc., with baseline resolution. In the analysis of acidic and neutral impurities of illicit heroin, relative standard deviation (R.S.D.) values of about 0.5% for migration times and 4–8% for areas and peak heights were achieved. In the same paper, the separation of heroin, heroin impurities, degradation products and adulterants, cocaine, benzoylecgonine, *cis*- and *trans*-cinnamoylcocaine was also reported.

In comparison with HPLC, MECC allowed the resolution of about twice as many peaks. However, HPLC was more sensitive. The MECC separation pattern was different from that of reversed-phase HPLC, proving that the two techniques may give ''orthogonal'' information.

Lurie and co-workers undoubtedly opened the way to the application of CE technology to the determination of drugs of forensic interest, but did not apply this technique to biosamples. As mentioned above, the first group active in this field was Thormann and co-workers, although more with clinical toxicology, than forensic toxicology purposes.

Wernly and Thormann in 1991 [8], using MECC in a fully aqueous borate–phosphate buffer pH 9.1 containing 75 m*M* SDS and a 75-μm I.D., 90-cm long fused-silica capillary, first reported the qualitative analysis of many abused drugs and metabolites in urine, including benzoylecgonine, morphine, heroin, 6-monoacethylmorphine (MAM), methamphetamine, codeine, amphetamine, cocaine, methadone, methaqualone and benzodiazepines. Detection was ''in capillary'' by a fast scanning UV spectrophotometer. Thus peak identification was based not only on the migration times, but also on the on-line recorded UV spectra of the peaks. Urine purification and concentration was by ''double mechanism'' SPE, as discussed above, allowing a sensitivity of about 100 ng/ml in the biological matrix. According to the authors, CE showed a sensitivity comparable to usual nonisotopic immunoassays and

could be proposed for confirmation testing, after the usual screenings by enzyme-immunoassays.

In another paper, MECC (50 m*M* SDS in phosphate–borate buffer pH 7.8) was used for rapid and high-resolution separation of barbiturates, namely barbital, allobarbital, phenobarbital, butalbital, thiopental, amobarbital, pentobarbital, with on-column multiwavelength detection (195 and 320 nm), achieving sensitivities in the range of the low μg/ml [37]. The authors concluded that this approach may be interesting for drug and metabolite investigations and for barbiturate identification in toxicological samples. The data indicated that some (including phenobarbital) but not all of the serum barbiturates investigated can be analyzed without extraction, while SPE prior to analysis was necessary for determining barbiturates in urine.

A validated quantitative analysis of barbiturates based on MECC [100 m*M* SDS in 10 m*M* borate, 10 m*M* phosphate pH 8.5–acetonitrile (85:15, v/v)] and UV detection at 214 nm, was published later by Ferslew et al. [38]. Serum and urine (2 ml) were extracted with commercial ready-to-use LLE tubes. Amobarbital, butabarbital, butalbital, pentobarbital, phenobarbital and secobarbital were completely resolved and determined with sensitivity of about 0.1 μg/ml and linearity from 3 up to 60 μg/ml. Precision of relative migration times was characterized by R.S.D. values between 0.7 and 2.7% (with the exception of phenobarbital which for unexplained reasons showed an R.S.D. of 6.2%), while quantitative R.S.D. values measured in serum ranged from 2.3 to 9.8% within day and from 2.6 to 8.6% between days. Forensic cases are also reported, in which vitreous humor and gastric content were analyzed successfully.

Despite the claimed lack of sensitivity of CE, after adequate sample preparation, MECC (75 m*M* SDS in phosphate–borate buffer pH 9.1) allowed the determination of 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), the major urinary metabolite of delta-9-tetrahydrocannabinol (THC), which is the active principle in marijuana and hashish [39]. Using basic hydrolysis of urine (5 ml) and SPE, it was possible to achieve the sensitivity of 10 ng/ml in the original sample. Again, the use of a fast scanning UV detector offered an additional opportunity of peak identity confirmation, based on spectral data.

By far the most dangerous class of drugs of abuse are opiates, and in this field CE has shown excellent analytical potential. After the first report by Wernly and Thormann [8] in which heroin, morphine, MAM and methadone were detected in urine, the same authors [40] showed that also the major urine metabolite of heroin and morphine, morphine-3-glucuronide, can be determined in this biological fluid by either CZE (12 m*M* sodium tetraborate, 20 m*M* disodium hydrogenphosphate, pH 9.8) or MECC (75 m*M* SDS in phosphate–borate buffer, pH 9.2), with a detection limit of 1 μg/ml (spectral UV analysis), after SPE with C_8-silica cartridges.

A screening method for opiates (morphine, heroin, codeine), amphetamine and caffeine in urine and serum was reported by Hyötyläinen et al. [41], by using MECC (quite surprisingly CZE did not provide an acceptable separation of the analytes) in an electrolyte system composed of 50 m*M* glycine and 50 m*M* SDS at pH 10.5. Short capillaries (50 μm I.D., 23 cm length) were used for fast screening (less than 2 min), and longer capillaries for quantification (50 μm I.D., 67 cm length). Detection was by UV absorption at 200 nm. The core of the paper, however, was the use of two carboxylic acids as markers of electrophoretic mobility, to determine ''migration indices'' of the analytes, which were used for the identification of the compounds, instead of the usual, but less precise, migration times. The marker technique, based on the use of two or more compounds of known electrophoretic mobility to calculate the effective field strength, the electroosmotic flow velocity and consequently the electrophoretic mobility of unknown compounds, allowed highly reliable identification in CZE [42]. This approach was then adapted to MECC, where the net mobility of an analyte is determined by its total mobility and the electrophoretic mobility while partitioned into the micelles. The authors replaced the electrophoretic mobilities with equations of retention indices of the marker compounds. In effect, the repeatability of absolute migration times of amphetamine, caffeine, codeine, heroin and morphine was good in water solution (R.S.D. values $<0.58\%$), but less satisfactory in urine (R.S.D. values $<1.03\%$) and in serum (R.S.D. values $<4.22\%$); on the other hand, R.S.D. values of migration indices for all the analytes, in every matrix were $<0.55\%$ and often below 0.1%.

Also benzodiazepines could be assayed by CE, as reported by Schafroth et al. [43], who determined the major urinary compounds of eight common benzodiazepines (flunitrazepam, diazepam, midazolam, clonazepam, bromazepam, temazepam, oxazepam and lorazepam) by MECC, using 75 m*M* SDS in pH 9.3 phosphate–borate buffer with small amounts of organic modifiers (2-propanol, methanol and/or acetonitrile). Again, after enzymatic hydrolysis and SPE with commercial ''double mechanism'' cartridges, the authors reported a sensitivity better than that of the current immunoassays, making CE a candidate tool for immunoassay confirmation.

In a more specific and quantitative study, Tomita et al. [44] used MECC for the simultaneous determination of nitrazepam and its two major metabolites (7-aminonitrazepam and 7-acetamidonitrazepam) in urine. After extraction from 2 ml of urine with C_{18}-silica cartridges and reconstitution of the extract with 50 μl of separation buffer, consisting of a mixture of 60 m*M* SDS–6 m*M* phosphate–borate adjusted to pH 8.5/methanol (85:15, v/v). Detection was by UV absorption at 220 nm. Under these conditions, excellent peak shapes and resolution of the three analytes were obtained, with fairly clean urine blanks. The reported detection limits were 100–200 ng/ml of analyte in spiked urine, with good linearity up to 10 μg/ml and good precision, in terms of both peak areas (R.S.D. values 1.7–8.0% within day and 2.0–7.7% day-to-day) and migration times (R.S.D. values 0.2–1.7% within day and 1.0–1.8 day-to-day).

As an alternative to MECC, Chee and Wan [28] used a CZE method with 50 m*M* phosphate buffer pH 2.35 (75 μm I.D. 60 cm long bare silica capillary) achieving, in only 11 min, the separation of 17 basic drugs, including amphetamine, methamphetamine, procaine, butacaine, medazepam, lidocaine, codeine, meclizine, diazepam, doxapram and methaqualone. Detection was by UV absorption at 214 nm. Under these conditions, according to a plain electrophoretic mechanism of separation, drugs having lower pK_a values and consequently less positive charge, showed higher migration times. However, the influence of other factors (molecular size, tendency to interact with the column and ability to form doubly charged species) hampered a clear correlation between pK_a values and migration times. The migration time R.S.D. values were, in general, less than 1% and peak-area R.S.D. values were between 1.5 and 4.3%.

The described method was applied to the analysis of plasma and urine, after chloroform–2-propanol (9:1) extraction and 40-fold concentration, achieving an overall sensitivity of about 0.45 μg/ml. Reportedly, CZE offered advantages over MECC for drug screening, consisting of simpler background electrolyte preparation and shorter analysis times. The main limitation was the inability to analyze acidic, neutral and basic drugs in a single run as in MECC.

Quite recently, CZE was reported to provide excellent quantitative determination of opiates, comprising pholcodine, 6-MAM, morphine, heroin, codeine and dihydrocodeine in urine, using a running buffer of 100 m*M* disodium hydrogenphosphate at pH 6 (Fig. 1) [45]. Electrokinetic injection with field-amplified sample stacking, after SPE of urine on ''double mechanism'' cartridges, allowed detection limits in the region of 4–9 ng/ml. Levallorphan was used as internal standard to limit imprecision inherent in the chosen injection method and the assay passed a careful validation procedure. The precision of electromigration times was 1.1% R.S.D. or less, resolution between adjacent peaks >2, plate number above 200 000; within-day and day-to-day repeatability was characterized by R.S.D. values in the range of 1 to 4%, when peak-area ratios were used. CZE results compared favorably with HPLC in the analysis of urine from a real user of dihydrocodeine and pholcodine.

CZE also proved superior to MECC for the simultaneous determination of methadone and its primary metabolite in urine. Molteni et al. [46] reported the CZE separation of the two compounds in 50 m*M* sodium tetraborate pH 9.3 in less than 10 min, with a sensitivity of about 20 ng/ml (or 2 μg/ml with direct injection of untreated urine) using a UV high-speed scanning from 195 to 320 nm. Urine samples were subjected to SPE with ''double mechanism'' cartridges. Under these conditions, R.S.D. values are reported to be 0.9% for retention times, and 6.0–6.4% for peak areas. Good agreement was found between results from CZE and gas chromatography–mass spectrometry.

Later, Lanz and Thormann [47] reported the characterization of the stereoselective metabolism of methadone and its primary metabolite by β-CD CE analysis of their enantiomers in urine.

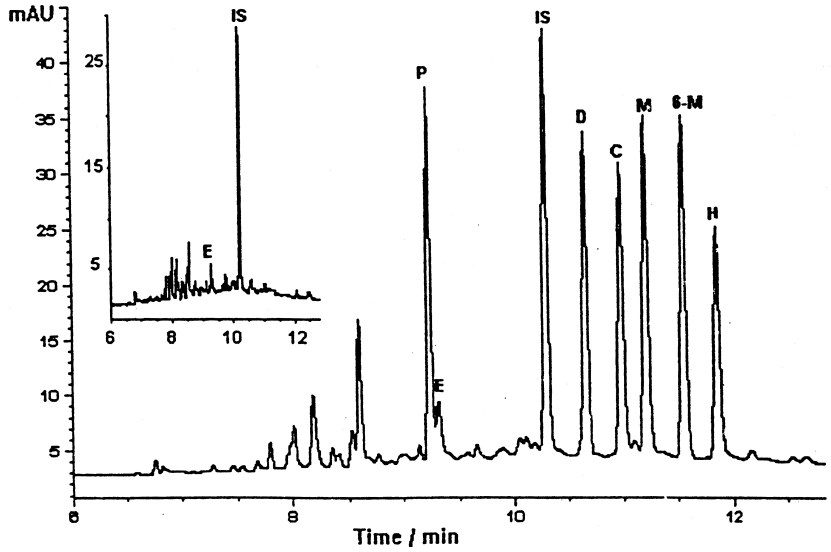

Fig. 1. Representative CZE electropherograms in 100 m*M* phosphate buffer pH 6.0 of urine spiked with six opiates: pholcodine (P), MAM (6-M), heroin (H), codeine (C), morphine (M), dihydrocodeine (D) and levallorphan (I.S.) as internal standard, after solid-phase extraction and electrophoresis with UV absorbance detection at 200 nm (for analytical conditions see text). An endogenous compound (E) with similar retention time to pholcodine, but still reliably distinguishable, was identified in blank urine (inset). From Ref. [45] with permission.

Hair analysis is gaining increasing popularity in forensic toxicology, as a tool for investigating past, chronic exposure to illicit drugs and, in this field, CE could offer clear advantages over current chromatographic techniques, due to the minimal need of sample mass for analysis, which in the case of hair can be a crucial point.

In the first report from our group, CZE was adopted for morphine and cocaine determination in hair, using a basic background electrolyte consisting of 50 m*M* borate, pH 9.2 [48]. Hair samples (about 100 mg) were first incubated overnight in 0.25 *M* HCl at 45°C, then the mixture was extracted by LLE before injection. Detection was either at 200 nm for the simultaneous analysis of cocaine and morphine or at the absorbance maxima of each analyte (for cocaine: 238 nm; for morphine: 214 nm) for higher selectivity (Fig. 2). Tetracaine and nalorphine were chosen as internal standards for cocaine and morphine, respectively. Excellent resolution and peak shape were obtained for both the analytes and the respective internal standards. The separations were highly efficient (up to 350 000 theoretical plates) and repeatable (migration time R.S.D. values: $<1\%$ within-day, $<3\%$ inter-days) and the quantitative determinations accurate and precise (within-day R.S.D. values in the range 3–5%). However, because of the tiny volumes of sample injected (a few nl) and the moderate concentration sensitivity of CE, the limit of detection in hair was acceptable (<0.2 ng/mg) only if the hair extracts were reconstituted with 10–20 μl of buffer, which was impracticable in a real situation. More recently, the optimization of injection by using a simple stacking technique has allowed the increase by about 10 times of the sample volumes loaded into the capillary, thus permitting the reconstitution of extracts with a larger volume of water (100 μl), without sacrificing the separation performance [49]. In the same paper, MECC has been tested in analyzing hair samples, using 100 m*M* SDS in 25 m*M* borate, 20% methanol. The sensitivity achieved was slightly worse than with CZE, but the selectivity was higher, due to the additional ''reversed-phase'' like separation mechanism with the SDS micelles.

Undoubtedly, chiral analysis is the field in which CE has most clearly shown a potential superior to existing techniques. The efficiency and selectivity but, particularly, ease, speed and economy of operation of CE can hardly be challenged by any chro-

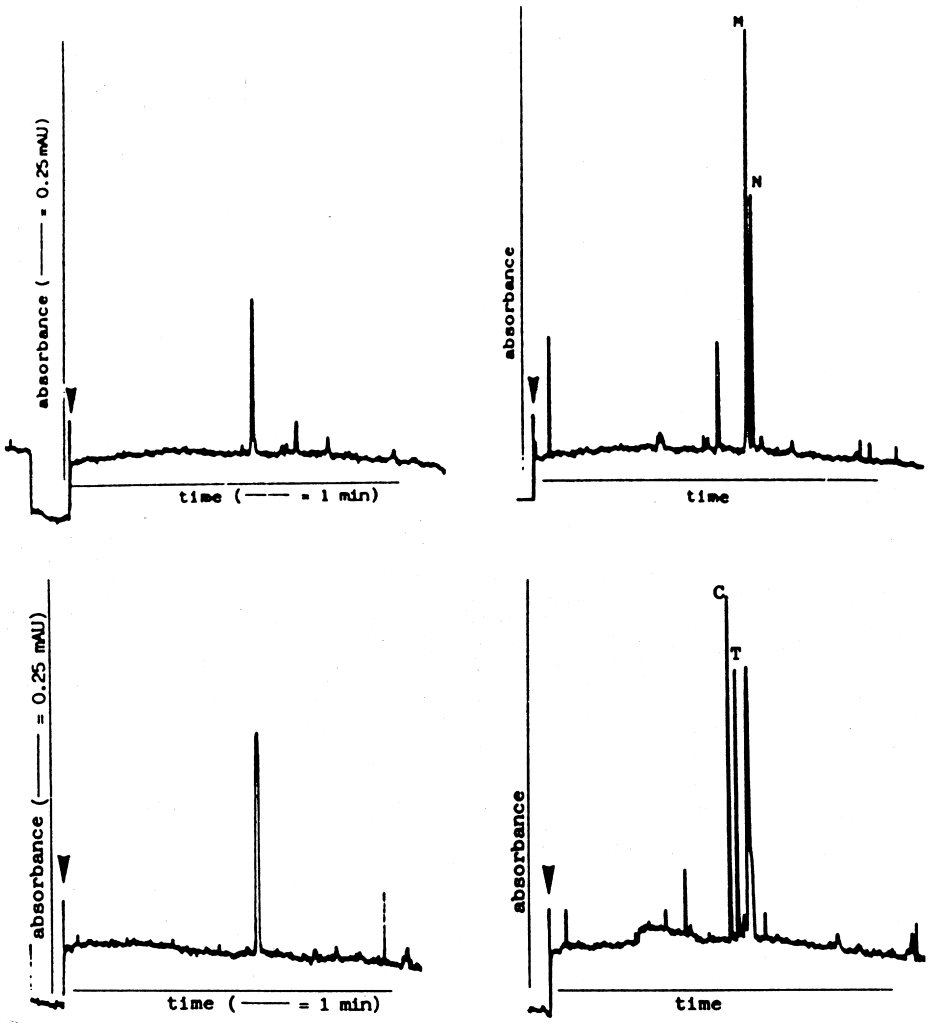

Fig. 2. Top: typical CZE electropherograms in 50 m*M* borate buffer pH 9.2 with UV absorbance detection at 214 nm of (left) a blank hair sample and (right) hair (75 mg) from a heroin user, containing morphine (M) at the level of 3 ng/mg (N=nalorphine, the I.S. used); bottom: typical CZE electropherograms in 50 m*M* borate buffer pH 9.2 with UV absorbance detection at 238 nm of (left) a blank hair sample and (right) hair (75 mg) from a cocaine user, containing cocaine (C) at the level of 4 ng/mg (T=tetracaine, the I.S. used). From Ref. [48] with permission.

matographic method. In CE, by far the most popular approach to chiral analysis for many therapeutic and forensic drugs is by using native or modified cyclodextrins (CD) in solution, which allows good selectivity and excellent efficiency, without any need for analyte derivatization (for a review see Ref. [50]).

The analysis of enantiomeric ratios of chiral drugs may be important for the investigation of synthetic routes of illicit drug seizures, as well as, in biological fluids, for understanding drug toxicity and metabolic precursors or products of given analytes. Notwithstanding several applications of chiral CE on pure compounds and clandestine preparations, this technique has rarely been applied to the analysis of drugs of forensic interest in biosamples.

To the best of our knowledge, the first application of chiral CE to analysis of drugs of forensic interest in biological fluids dates back to 1993, when Aumatell and Wells [51] published the simultaneous CE analysis of the optical isomers of racemethorphan

and racemorphan in urine, after reversed-phase SPE. The method was based on 60 m*M* β-CD as the chiral selector, in 50 m*M* borate pH 9.05, 50 m*M* SDS, 20% 1-propanol; detection was by UV absorption at 200 nm. Complete resolution of levomethorphan (L-3-methoxy-*N*-methyl-morphinan, narcotic analgesic, not commercially available) from dextromethorphan (D-3-methoxy-*N*-methyl-morphinan, allowed antitussive) and levorphanol (L-isomer of dextrorphan, narcotic analgesic, banned for athletes) from dextrorphan (metabolite of dextromethorphan) and of all these compounds from ethylmorphine, the internal standard, was achieved in less than 50 min, with a sensitivity of about 20 ng/ml in urine. Thus, without derivatization, the differentiation between two enantiomers, one of which, dextrorphan, is the metabolite of an allowed drug (dextromethorphan) and the other, levorphanol, is a banned drug, could be achieved by chiral MECC in urine extracts.

Varesio and Veuthey [52] reported the simultaneous chiral separation of amphetamines, including amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDE), in urine using 20 m*M* (2-hydroxy)-propyl-β-CD, as the chiral selector, in 200 m*M* phosphate buffer pH 2.5. Detection was by UV at 200 nm wavelength. All analytes were baseline resolved with excellent enantiomeric resolution in 25 min. Migration time R.S.D. values were 0.3–0.4%, while peak area R.S.D. values were 4.3–9.1% (with internal standardization). Sensitivity was better than 0.5 μg/ml in urine (the cut-off established by most regulatory authorities), after either LLE or SPE, but the latter method gave cleaner extracts.

The same panel of analytes plus ephedrine, was also completely resolved by using underivatized β-CD 20 m*M* in 150 m*M* phosphate pH 2.5, with a possibility of UV spectral analysis, and the method was applied to urine and hair extracts, after simple LLE, with good reproducibility [53,54]. However, while amphetamine concentrations present in urine (>0.2 μg/ml) could easily be determined, a field-amplified sample stacking technique had to be applied to achieve the sensitivity required for hair analysis (<0.5 ng/mg).

3.2. Analysis of other drugs and toxicants of potential forensic interest

Almost all the existing drugs and chemicals may have a forensic relevance, in cases of intentional or accidental intoxication. Thus CE, because of its high versatility and broad analytical spectrum, may be a valuable analytical tool.

Tricyclics are a wide group of drugs widely used as antidepressants (e.g., amitriptyline), antipsychotics (e.g., chlorpromazine) and cardiac antiarrhythmics (amiodarone). Since these drugs may produce fatal intoxications in humans and can be used for illicit treatment of food animals (to improve the quality of meat) their determination in biological samples is particularly important.

Aumatell and Wells [55] described a MECC method for the determination of as many as 26 tricyclic drugs in human and animal urine after LLE with *n*-hexane. The MECC system was based on 10 m*M* sodium taurodeoxycholate, a bile salt, in 40 m*M* sodium tetraborate pH 9.5. A 50 μm I.D., 50-cm long fused-silica capillary was used along with a UV absorbance detector operated at 240 nm. Under these conditions, starting from 10 ml of sample, the limits of detection for all analytes were between 4 and 86 ng/ml. No further validation was reported.

A quantitative MECC determination of a smaller number of tricyclic drugs, namely desipramine, nortriptyline, doxepin, imipramine and amitriptyline, in serum was published with validation data by Lee et al. [56]. The separation buffer was 37.5 m*M* phosphate pH 8.0; the micellar agent was 25 m*M* dodecyltrimethylammonium bromide, a cationic surfactant, which reversed the electroosmotic flow and consequently needed the reversal of the potential (−25 kV); 2 *M* urea was added to tune partitioning of analytes in the micelles. Detection was by UV absorption at 254 nm. One milliliter of serum or plasma was added with the internal standard and with 1 ml of 2 *M* NaOH, then extracted with 5 ml of hexane–isoamyl alcohol (99:1, v/v) and back-extracted into the aqueous phase by adding 100 μl of 0.1 *M* HCl. The aqueous layer was dried and the residue reconstituted in 20 μl of water for CE analysis. Under these conditions, detection limits as low as 5–10 ng/ml could be attained. Linearity was

successfully tested from 50 to 250 ng/ml, corresponding to the therapeutic range of the drugs. R.S.D. values of retention times were <0.8% within-run and <2.0% between-runs, while those of the peak areas were <6–8% in both within- and between-runs.

Due to their narrow therapeutic range, ultrashort and short acting barbiturates are to be determined in circulating fluids for either therapeutic drug monitoring or, in case of fatalities, forensic toxicology purposes.

Thiopental, used for anaesthetic and preanaesthetic medication and treatment of severe brain traumatic injuries, was determined by Meier and Thormann [57] by using MECC and reversed-phase HPLC, after LLE of serum (0.5 ml) with *n*-pentane. The MECC buffer was composed of 50 m*M* SDS, 9 m*M* sodium borate and 15 m*M* sodium dihydrogenphosphate, pH about 7.8. UV detection was at 290 nm. Carbamazepine was used as internal standard. Thiopental, its isomer and carbamazepine were nicely separated in about 8 min, with a linearity range from 2 to 60 μg/ml, and good reproducibility of both migration times (R.S.D. values=0.95%) and area ratios thiopental/carbamazepine (R.S.D. values=2.08%). A comparison of CE and HPLC results from the analysis of 66 patient samples was acceptable but, quite surprisingly, showed a better correlation without than with the use of internal standard. No explanation was given by the authors.

On the other hand, CZE was also successful in the determination of a short-acting barbiturate, pentobarbital, in serum, as reported by Shihabi [58]. The electrophoretic separation was carried out in 300 m*M* borate buffer pH 8.5 with detection at 254 nm. Both serum deproteinization with acetonitrile and extraction with chloroform gave suitable extracts for analysis. The assay was linear between 10 and 100 μg/ml, with within-run R.S.D. values for migration times of 0.8% and for peak heights of 1.4% (relative to an internal standard). Excellent correlation with HPLC was reported.

Need for constant monitoring of circulating drug concentrations with potential forensic implications is typical for antiepileptics. The simultaneous, quantitative determination of ethosuccimide, phenytoin, primidone, phenobarbital and carbamazepine in human plasma was achieved by MECC with a fast scanning UV detector in the wavelength range of 195–320 nm (valproic acid was separated but not extracted) [59]. Plasma was added with hexobarbital, as an internal standard, and subjected to LLE using ethyl acetate. The separation buffer was composed of 25 m*M* phosphate containing 50 m*M* SDS at pH 7.0. Under the described conditions, good linearity was reported for all the analytes within the individual therapeutic concentration ranges. The analytical precision was characterized by within-day R.S.D. values <0.5% for migration times and <4% for peak areas, except for ethosuccimide whose R.S.D. values varied from 2.5 to 8.3%. On-line recorded spectra of the peaks of the analytes were found to be superimposable on spectra of reference standards.

Theophylline is a well-known, potent bronchodilator and respiratory stimulant, but is potentially toxic and shows a narrow therapeutic window; therefore, it has long since attracted the attention of clinical toxicologists and is usually subjected to therapeutic drug monitoring. Because of the relatively high therapeutic concentrations (5–20 μg/ml), serum samples can be simply deproteinized [22,24] with organic solvents and injected; even direct injection of serum or saliva was proposed as an alternative to SPE and LLE (SPE was needed for urine) [60]. The latter approach was made possible by using MECC (75 m*M* SDS in phosphate–borate buffer pH 9) which prevented protein interferences on the separation. Under these conditions, theobromine, caffeine, paraxantine, theophylline, 7-methylxantine, 3-methylxantine, 3-methyl uric acid, 1-methyl uric acid, 7-methyluric acid and uric acid could be resolved and detected by multiwavelength UV spectrophotometry. With direct sample injection, migration time R.S.D. was about 0.6% and peak area R.S.D. values were 5–7%, but the method lacked a careful validation. Theophylline and caffeine were determined in serum samples from patients and the results compared favorably with nonisotopic immunoassays currently in use in clinical chemistry laboratories for theophylline, but for caffeine an unexplained overestimation of MECC was reported.

In a more carefully validated quantitative paper, Lee et al. [61] reported the use of MECC (80 m*M* SDS in 25 m*M* phosphate pH 8) with UV absorbance

detection at 274 nm for theophylline and caffeine determination in serum and plasma (100 μl), after extraction with ethyl acetate. Linearity was tested successfully from 5 to 50 μg/ml, the within-run migration time R.D.S. values were <1% and those of peak areas were 4.81, 2.98 and 3.11% for theophylline, paraxanthine and caffeine, respectively; the between-run R.S.D. values of migration times were 2.9–3.9% and those of the peak areas were 5.8–6.5%.

An alternative approach to quantitative analysis of serum theophylline, based on CZE with UV absorbance detection at 272 nm, after serum deproteinization with methanol and internal standard addition (8-Cl theophylline), has been reported by Tagliaro et al. [22]. Sensitivity (2 μg/ml), linearity (up to 120 μg/ml) and quantitative precision (R.S.D. values= 4.9 and 6.3% within-day and between-days, respectively) were adequate for use in clinical chemistry and toxicology laboratories. A comparison of the performances of the two methods showed quantitative precision data absolutely comparable, despite the use of an internal standard in the latter, which, however, was carried out with a manual electropherograph. This seems to indicate that in CE, as in HPLC, with careful sample handling one can achieve analytical precision levels acceptable to meet the standards required for the analysis of biological samples. Of course, internal standardization can protect against random mistakes in the extraction procedure, which may be missed using external standards only.

Other therapeutic drugs, without a high intrinsic toxicological potential, are susceptible to forensic toxicology controls, as they can be illicitly used as doping agents in sports, and thus are banned or controlled by the International Olympic Committee.

Ephedrine is a sympathomimetic drug stimulating the central nervous system as well as the vasomotor system and respiratory function, and consequently is susceptible to abuse to improve physical performance [13]. Ephedrine and its metabolite norephedrine were simultaneously determined in human urine by CZE in 50 m*M* phosphate buffer pH 9.5 containing 1% acetonitrile, which was added for finely tuning the separation (probably by reducing the electroosmotic flow). Detection was by UV at 210 nm and injection was carried out after urine dilution. The limit of detection was 0.8 μg/ml of ephedrine; for concentrations between 3 and 9 μg/ml, R.S.D. of determinations was reported as ≤3.7%. In a further paper from the same group [62], improved analytical conditions (50 m*M* phosphoric acid adjusted to pH 9.7, 10% acetonitrile) allowed the determination of ephedrine, pseudoephedrine, norephedrine, norpseudoephedrine, methylephedrine and methylpseudoephedrine in urine, after 1:5 sample dilution with water. Unfortunately, quantitative validation was superficial.

β_2-Adrenergic agonists are common drugs used as bronchodilators and tocolytic agents, but are also susceptible to abuse in sports to improve respiratory efficiency and, on the basis of a side effect on energy repartition between fat and muscle, to promote muscle mass. The same effect is illicitly used to improve the quality of meat of food animals. For these reasons, there is a great interest in the determination of this class of drugs in biological specimens.

Clenbuterol is the most important drug subjected to illicit use because its high potency allows administration at very low doses. Notwithstanding the very low concentrations of this drug (≥0.2–0.5 ng/ml are present in urine), which is the most widely used sample for β_2-agonist monitoring, a CZE method with UV absorbance detection proved capable of determining clenbuterol in this matrix [63]. LLE of 10 ml of alkalinized urine with chloroform, evaporation of the solvent and reconstitution with 50 μl of water was the sample preparation procedure. Separation was carried out in 25 m*M* citrate pH 4.5–6.0 and detection was at 210 nm wavelength (with the possibility of spectral analysis for high concentration samples). The assay proved linear from 0.5 to 3.0 ng/ml; R.S.D. values obtained from triplicate analysis were <0.5% for migration times and <4.0% for normalized areas. A CE analysis of calf urine was in agreement with results from immunoassay and gas chromatographic–mass spectrometric analyses.

β-Adrenergic blocking agents are widely in use for the treatment of hypertension, cardiac disorders (angina, arrythmias etc.) and migraine, but because of their inherent sedative effect, they are also abused as doping agents in sports. Due to the high number of different molecules on the market with different physicochemical characteristics (e.g., lipophilicity),

the simultaneous determination of the major components of this class of drugs is extremely difficult. The ability of MECC to deal with compounds within a wide range of polarity allowed the separation of ten parent β-blockers (acebutolol, nadolol, timolol, atenolol, metoprolol, oxprenolol, pindolol, alprenolol, labetalol, propranolol) in less than 20 min, as reported by Lukkari et al. [64]. Urine samples were just diluted 1:2 with water, added with an internal standard (2,6-dimethylphenol), filtered and injected. The MECC buffer was composed of 80 m*M* phosphate pH 7.0 containing 10 m*M* *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB), a cationic surfactant. Consequently, separation was performed at inverted polarity (injection at cathode). Detection was by UV absorbance at the fixed wavelength of 214 nm. Notwithstanding excellent separation and acceptable quantitative repeatability (R.S.D. values between 2 and 7%), the assay sensitivity, 10–20 μg/ml was poor for application in real cases. The analysis of these drugs (excluding labetalol) in serum required minor adjustments of the separation buffer (80 m*M* phosphate pH 6.7 containing 15 m*M* CTAB), also the internal standard was replaced by ephedrine [65]. Serum treatment required enzyme hydrolysis (*Helix pomatia*), protein precipitation with acetonitrile and filtration. Under these conditions, acceptable precision (R.S.D. values between 4.5 and 15.8%) and linearity were found over the range 75–300 μg/ml, but sensitivity was clearly inadequate. However, after a preconcentration step (e.g., acetonitrile evaporation), it was possible to measure therapeutic levels of propranolol in serum.

A comparison of ion-pair HPLC and MECC, both using CTAB, in the analysis of β-blockers in human biological fluids showed that HPLC was better in terms of sensitivity (0.1–0.7 μg/ml with HPLC in comparison to 1–50 μg/ml with CE), but MECC was superior for ease of sample preparation, efficiency and resolution power [66].

Also, an enantioselective determination of oxprenolol and its metabolites in urine based on hydroxypropyl-β-CD CZE–UV in human urine, after LLE with ethyl acetate, was reported by Li et al. [67].

Diuretics are widespread therapeutic drugs which can be misused by athletes to decrease body weight or to mask the intake of doping agents. For this reason they are totally banned by the International Olympic Committee.

Jumppanen et al. [68] reported a screening procedure for this heterogeneous group of drugs based on: (a) CZE at pH 10.6 in 60 m*M* 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) for diuretics containing sulfonamide and/or carboxylic groups and (b) CZE at pH 4.5 in 70 m*M* acetate–500 m*M* betaine for compounds containing amine groups. Detection was by UV absorption at 220 and 215 nm. At pH 10.6, metyrapone and caffeine (coelution), triamterene and amiloride (coelution), clopamide, chlorthalidone, ethacrynic acid, probenecid, bumetadine, bendroflumethiazide, furosemide, trichlormethiazide, beniazidezthiazide, hydrochlorothiazide, dichlorphenamide, chlorothiazide and acetazolamide migrated in this order. At pH 4.5 the migration pattern was: amiloride, triamterene, metyrapone and caffeine. After reversed-phase SPE, the detection limit for hydrochlorothiazide was 0.1 μg/ml. All compounds could be confirmed by gas chromatography–mass spectrometry.

Hypoglycemic drugs can be abused in order to mimic syndromes associated with pathological insulin secretion. The separation of some major sulfonylurea compounds, including chlorpropamide, glyburide, glipizide, tolazamide, acetohexamide and tolbutamide (plus an internal standard), in urine, after SPE extraction, was achieved in less than 8 min by MECC with a buffer composed of 75 m*M* sodium cholate in 5 m*M* borate–5 m*M* phosphate pH 8.5 [100]. Using 150 m*M* SDS as micellar agent also provided complete resolution of the analytes, but in a longer run (about 10 min). Detection was carried out either at a single wavelength of 200 nm or with a scanning UV spectrophotometer at 200–350 nm. The reported sensitivity was about 50 ng/ml for the individual drugs, precision was characterized by R.S.D. values of 0.89% for migration times and 8.49% for peak areas in within-run tests; only slightly worse figures were reported for between-run precision.

Paraquat (1,1′-dimethyl-4,4′-bipyridylium salt) and diquat (1,1′-ethylene-4,4′-bipyridylium salt) are widespread weedkillers throughout the world, but are extremely toxic to man. Both have been causes of suicidal, rarely homicidal, and accidental poisonings, and due to the composition of commercial products

containing mixtures of the two compounds, often they are to be simultaneously determined in biological fluids. After extraction from serum with reversed-phase disposable cartridges, paraquat and diquat were separated by CZE in 10 m*M* glycine–HCl buffer in 10 min and simultaneously detected by on-column UV absorbance at 200 nm wavelength [69]. Precision was tested at 0.5 and 2.5 μg/ml, obtaining R.S.D. values of 4.3 and 1.7% for paraquat and 0.5 and 2.5% for diquat, respectively.

Amatoxins (α- and β-amanitins) are the main toxins of the mushrooms of genus *Amanita* (*Amanita phalloides, Amanita verna, Amanita virosa*) and are among the most potent toxins for man (LD_{50}: 0.1 mg/kg). Due to the nonspecific clinical symptoms associated with amanita intoxication, which is frequently lethal, great attention has been devoted to its early diagnosis by analytical means. Unfortunately, the peptidic structure of these compounds (bicyclic octapeptides), the thermal instability and the low concentrations to be detected in biological fluids pose analytical problems still not satisfactorily resolved. CE is widely recognized as an analytical tool providing high efficiency separations in the liquid phase of peptide compounds, and was tested for amanitin determination by Brüggemann et al. [70]. The authors used plain CZE in 100 m*M* phosphate pH 2.4 and UV absorbance detection at 214 nm (or by recording absorbance spectra in the range 190–350 nm). The method proved highly efficient and acceptably linear (1–1000 μg/ml) and reproducible (R.S.D. values of peak heights <7.7%), but dramatically insensitive for meeting the sensitivity needs of clinical diagnosis (a few ng/ml in urine). However, it could be successfully applied to the assay of amanitins in toadstool extracts, with the possibility of spectral confirmation of peak identity. Despite the lack of sensitivity of the method, the authors, in the last part of the paper, struggled to identify α-amanitin in simply 1:1 diluted urine from intoxicated subjects, without a known reference method, by applying, in questionable modes, standard addition and spectroscopic peak identification methods. We regret the need to point out that we disagree completely with their conclusions, which seem to support the possibility of detection of α- and β-amanitin in patients' urine by CZE–UV with direct sample injection. The target concentration to be measured is, unfortunately, about three orders of magnitude lower than that allowed by the described CZE method.

Styrene is widely used in industry and is potentially toxic to the central nervous system, liver, lungs and kidneys. Its biological monitoring is based on the determination of major metabolites phenylglyoxylic acid (PGA) and mandelic acid (MA). Simon and Nicot [71] reported a simple CZE determination of the two analytes in C_8 coated capillaries with 40 m*M* sodium formiate pH 3.7, 10% 2-propanol, as separation electrolyte, with UV absorbance detection either at 255 or at 210 nm, for PGA and MA, respectively. Urine samples were simply tenfold diluted in acetonitrile, centrifuged and directly injected. The method was successfully validated in comparison with supercritical fluid chromatography.

3.3. Metabolism studies

The ability of CE in producing high efficiency separation of a wide spectrum of nonpolar, polar as well as ionic analytes in aqueous phase and at room temperature, thus avoiding thermal degradation, volatility and derivatization problems, and its compatibility with direct sample injection make this technique an almost ideal tool for drug metabolism studies.

To this purpose, to obtain the characterization of metabolite structure, the separation step must be followed by highly informative detection techniques, including UV spectroscopy and, above all, mass spectrometry (MS), particularly in the MS–MS configurations.

CE has often been applied to study drug metabolism in either CE or MECC modes, particularly to study phenotypic metabolic variants. This subject, however, is on the borderline of the scope of the present review.

In Table 1, examples are displayed of the possibilities offered by CE in the study of metabolic biotransformations of drugs of potential forensic interest. For more detailed information on the strategies for monitoring drug metabolism by CE techniques, the readers are referred to recent reviews and papers on this subject [72–75].

Table 1
Papers on metabolism studies of drugs of forensic interest using CE

Drug metabolites	Pretreatment	Injection	Separation	Detection	Ref.
Flurazepam metabolites; sulfonamides	Hydrolysis, LLE	Hydrodynamic	CZE: 15 or 0.2 m*M* ammonium acetate adjusted to pH 2.5 or 1.3 with TFA, 15% methanol capillary: uncoated, 75 μm I.D., 100 cm long	UV at 254 nm; CE–atmospheric pressure ionization–triple quadrupole MS	[88]
Substituted purines	Direct injection, LLE, SPE	Hydrodynamic	MECC: 75 m*M* SDS, 6 m*M* borate, 10 m*M* phosphate, pH≅9 capillary: uncoated, 75 μm I.D., 90 cm long	Scanning UV with spectra recording: range 195–320 nm	[60]
Caffeine metabolites (acetylator phenotyping)	Direct injection, LLE	Hydrodynamic	MECC: 70 m*M* SDS, phosphate–borate, pH 8.43 capillary: uncoated, 50 μm I.D., 72 cm long	UV at 254 nm	[93]
Caffeine metabolites (acetylator phenotyping)	LLE	Hydrodynamic	MECC: 70 m*M* SDS, 16.2 m*M* borate, 16.2 m*M* phosphate, pH 8.6 capillary: uncoated, 75 μm I.D., 70–110 cm long	Scanning UV with spectra recording: range 195–320 nm	[94]
Dextromethorphan and dextrorphan	Direct injection, hydrolysis	Hydrodynamic	CZE: 175 m*M* borate, pH 9.3 capillary: uncoated, 50 μm I.D., 72 cm long	UV at 200 nm	[14]
Haloperidol metabolites	Reversed-phase SPE	Hydrodynamic	CZE: 50 m*M* ammonium acetate, 10% methanol, 1% acetic acid, pH 4	UV at 214 nm	[95]
Haloperidol metabolites	Reversed-phase SPE	Hydrodynamic	CZE: 50 m*M* ammonium acetate, 10% methanol, 1% acetic acid, pH 4.1 capillary: uncoated, 50 μm I.D., 65 cm long	Scanning UV with spectra recording: range 195–320 nm; CE–MS and CE–electrospray-collision induced dissociation–MS	[96]
Mephenytoin and dextromethorphan metabolites	Enzymatic hydrolysis	Hydrodynamic	MECC: 75 m*M* SDS, 6 m*M* borate, 10 m*M* phosphate, pH 9.2–9.3 CZE: 140 m*M* borate pH 9.4 capillary: uncoated, 75 μm I.D., 90–105 cm long	Scanning UV with spectra recording: range 195–320 nm	[15]
Theophylline metabolites	Reversed-phase SPE	Hydrodynamic	MECC: 100 m*M* borate, 100 m*M* phosphate, pH 8.5 with 200 m*M* SDS in a ratio 7:12, final pH 6.5 capillary: uncoated, 75 μm I.D., 67 cm long	As in ref. [60]	[97]
Dihydrocodeine metabolites	Direct injection or double mechanism SPE; hydrolysis	Hydrodynamic electrokinetic	MECC: 75 m*M* SDS, 6 m*M* borate, 10 m*M* phosphate, pH 9.2 capillary: uncoated, 75 μm I.D., 70 cm effective length	UV at 213 nm; scanning UV with spectra recording: range 195–320 nm	[98]
Dihydrocodeine *O*-demethylation	SPE; hydrolysis	Hydrodynamic	MECC: 75 m*M* SDS, 6 m*M* borate, 10 m*M* phosphate, pH 9.2 capillary: uncoated, 75 μm I.D., 70 cm effective length	UV at 213 nm	[99]

4. Advanced CE separation and detection techniques

4.1. Alternative separation methods

Although historically isotachophoresis was extensively used for analysis of drug and toxicants, in recent capillary configurations (CITP) this technique has not gained great popularity in analytical toxicology (for a general review see Ref. [76]).

The comparative use of MECC, CZE and CITP for rapid diagnosis of medical drug intoxications was reported by Caslavska et al. [77]. Salicylate, paracetamol and antiepileptics were analyzed in serum and urine. In MECC, 75 m*M* SDS in borate–phosphate buffer pH 9.1 was employed, whereas CZE was carried out with 33 m*M* phosphate buffer pH 8.3; 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)ethanesulfonic acid and histidine (pH 6.0). In cases of high drug concentrations, body fluids could be injected directly or with simple pretreatments, such as dilution (urine) or ultrafiltration (serum). However, in cases of drugs at the low μg/ml level, extraction and concentration were found necessary. The results showed that MECC and CZE were easier to use, whereas CITP required a more complex selection of buffers. This is probably the main reason that has hampered a more widespread application of CITP in analytical toxicology laboratories. On the contrary, isotachophoresis has become a promising tool for sample pretreatment followed by a CZE separation step (in the same column or in two different columns), allowing high concentration factors to be achieved with consequent improvement in sensitivity [31]. However, very little validation can be found in most of these papers, showing, in our opinion, that this approach is still not ready for application in toxicology laboratories.

On the other hand, the CITP determination of some cardiovascular drugs (amiloride, metoprolol, deacetylmetipranolol, labetalol and furosemide) in human serum and urine was reported by Sádecká and Polonský [78]. Amiloride and β-blockers were separated by cationic isotachophoresis in a system 10 m*M* sodium morpholinoethanesulfonate buffer (pH 5.5)–glutamic acid, furosemide by anionic isotachophoresis in 10 m*M* histidine HCl (pH 6.2)–morpholino-propanesulfonic acid. Sample pretreatment was carried out by reversed-phase SPE. Detection was by conductivity measurement. Under these conditions, the overall limits of determination ranged from 32 to 46 ng/ml for urine and from 39 to 46 ng/ml for serum. The relative standard deviations varied from 0.1 to 5.6% in the range of concentrations tested, 100–2500 ng/ml.

A new separation mode, which was proposed for the direct analysis of drugs in urine is based on mixed polymer networks. In this approach, a two-phase system of polyethylene oxide–polydextran in 32 m*M* 6-aminocaproic acid–18 m*M* adipic acid pH 4.5–5% methanol in uncoated capillaries was found to produce excellent separations of pharmaceuticals such as cimetidine, famotidine, diltiazem and prazosin, including degradation products [79]. The separation mechanism seemed to resemble electrochromatography, by interaction of the analytes with the polyethylene oxide polymer residing in the capillary. Precision of migration times and peak heights was evaluated, obtaining, under the optimal conditions, R.S.D. values of 0.9 and 1.7%, respectively. By addition of CDs, chiral selectivity was also achieved. Also, the mixed polymer network system was found suitable for direct injection of urine.

4.2. High sensitivity detection methods

Fluorescence detection is universally known to be extremely sensitive and selective, and consequently useful for analysis of complex biological matrices. In CE, due to problems in concentrating enough radiation energy inside a tiny capillary, fluorescence detection is generally performed using laser sources for excitation (LIF), instead of more traditional lamps. Unfortunately, the choice of wavelengths emitted by lasers (at affordable costs) is limited, and this is the main limitation of LIF application to drug analysis. However, when LIF can be applied, the sensitivity limits of CE can be improved by a factor of about 1000 or more over UV absorbance detection.

An excellent application can be found in a recent paper by Hempel and Blaschke [80], who reported a direct highly sensitive determination of zolpidem, a new sleep inducer, and its four metabolites, in urine. The method was based on a simple CZE separation

(50 m*M* phosphate pH 5.6) with LIF detection, using for excitation a He–Cd laser emitting at 325 nm wavelength. Urine had just to be enzymatically hydrolyzed and injected in hydrodynamic mode. The sensitivity was about 2 ng/ml, while quantitative reproducibility was described by R.S.D. values around 3.5 and up to 19.7% at the limit of quantitation, which was placed at 10 ng/ml.

LIF detection also allowed direct CE determination of LSD in blood [81] which, because of its extremely low concentrations, represents one of the most challenging toxicological analyses in biosamples.

When the molecular structure is not adequate for providing natural fluorescence, analytes can be derivatized with fluorophores. A recent example is given by Páez et al. [82], who reported 3 amol as the limit of mass detection (three orders of magnitude lower than in gas chromatography–mass spectrometry) and 3 n*M* in terms of concentration for the CZE–LIF analysis of amphetamine, after derivatization with fluorescein isothiocyanate. In this case, the excitation radiation was provided by an Ar-ion laser emitting at 488 nm.

A wider spectrum of excitation wavelengths is provided by xenon-arc lamp irradiation, which can be tuned, easily extending into the UV region, according to the fluorescence spectra of the analytes. This detection method proved successful in combination with CZE separation for the determination of diuretics banned in sport (amiloride, triamterene, bendroflumethiazide and bumetanide) [83]. At pH 8, in less than 8 min a complete separation of the four analytes was achieved with sensitivities between 0.09 (triamterene) and 3.6 μ*M* (bumetanide), and with high selectivity allowing direct injection of urine. Excitation radiation wavelengths were in the range from 272 to 382 nm for the different analytes.

An original application of CE–LIF for high sensitivity drug analysis is its use in combination with a competitive immunoassay. This approach, proposed by Chen and Evangelista [84] is based on a competitive immunoassay with a fluorescent tracer. CZE–LIF was used to separate the free from the antibody bound fraction. Because of the separation of the free tracers by CE, multiple analytes could be determined simultaneously in urine, as demonstrated by the authors for morphine and phencyclidine with sensitivities in the n*M* range. The same strategy was later used by Steinmann et al. [85] for the immuno-CE–LIF analysis of theophylline in serum. The authors preferred MECC (with SDS) to CZE to hinder capillary wall poisoning by sample proteins, which could perturb the assay. Results from immuno-CE–LIF correlated favorably with a reference direct injection MECC analysis of theophylline.

Quite surprisingly, to the best of our knowledge, another highly sensitive technique such as amperometric detection (AD) has not been applied to the CE analysis of forensic drugs. In reality, the problem of coupling the high potential–high current CE separation with the amperometric cell in which very low faradic currents are to be measured has not yet been completely resolved, and is possible only by laboratory-made devices. This fact has probably hampered the marketing of reliable instrumentation and consequently the penetration of CE–AD in the field of toxicological analysis, where analytical reliability is of the highest importance.

However, due to the potential of this technique shown in analytical pharmacology and biochemistry [86], we believe that in the near future CE–AD will be used also in the fields of forensic toxicology, in which electrochemical detection has proved a simple and low cost method to achieve the highest analytical sensitivity (e.g., opiates, cannabinoids, ring-substituted amphetamines, etc.).

4.3. CE–mass spectrometry

The fundamental role of mass spectrometry (MS) in modern analytical toxicology is indisputable. Consequently, the extreme interest of forensic toxicologists for coupling separation techniques with MS is easily understandable. After the spread of GC–MS in almost any toxicology laboratory, becoming a kind of a ''gold standard'' in this field, and the more recent introduction of HPLC–MS, CE–MS seems at present to be ''on the launching pad''.

The development of on-line CE–MS, started in the late 1980s, has undergone a steady progression until the recent introduction of commercial CE instrumentation ''ready'' for coupling with mass spectrometers. Several ionization methods have been proposed for CE–MS, but electrospray is the one

which has gained most success (CE–ESI-MS). Although some limitations regarding the selection of CE electrolyte composition and the concentration sensitivity of the CE–MS hyphenation have not yet been completely overcome, several applications have started to appear, particularly in the biological sciences, drug metabolism and environmental analysis. A review of the recent instrumental approaches to CE–MS, including applications, has been published by Cai and Henion [87].

Unfortunately, very little has appeared so far in the field of forensic drug analysis. To the best of our knowledge, the first report concerning analysis of drugs of forensic interest by CE–MS dates back to 1991, when Johansson et al. [88] reported the determination in urine of sulfonamides and benzodiazepines by CZE (in 15–20 m*M* ammonium acetate solutions, at different pH values, containing 15–20% methanol) with atmospheric pressure ionization–MS. Samples were subjected to LLE prior to injection. A detection limit of about 500 ng/ml in urine of the major metabolite of flurazepam (*N*-1-hydroxyethylflurazepam) was reported.

In order to improve the concentration sensitivity of CE–MS, which appears to be the main obstacle to the application in a forensic toxicology environment, the coupling of in-capillary isotachophoresis with CZE–MS was proposed by Lamoree et al. [89] for the highly sensitive analysis of clenbuterol and salbutamol in calf urine, with detection limits in the ng/ml range. Biological samples were first subjected to immunoaffinity extraction and SPE. The CZE buffer was also the leading buffer in the isotachophoretic step, being composed of 50 m*M* ammonium acetate pH 4.8–methanol (1:4, v/v), while the terminating buffer was composed of 50 m*M* β-alanine pH 4.8–methanol (1:4, v/v). Samples were dissolved in the CZE buffer and hydrodynamically injected into the capillary which was filled with leading buffer. After injection, the capillary inlet was placed in the anode vial containing the terminating buffer. When the voltage was applied, the sample zone migrated towards the cathode, which is the electrospray tip. During the migration, the sample components were separated according to their electrophoretic mobility and focused according to the concentration of the leading buffer. In order to improve the focusing step, a hydrodynamic flow was introduced towards the anode. After analyte focusing, the voltage was turned off, the capillary inlet placed in the leading buffer and the potential started again for carrying out a CZE separation. A single-quadrupole MS equipped with an electrospray interface was used in the positive ion mode.

Also, we can mention the CE chiral separation with ion-spray MS detection of terbutaline and ephedrine in urine, reported by Sheppard et al. [90]. Separations were performed with 5 m*M* heptakis(2,6-di-*O*-methyl)-β-CD in 5 m*M* phosphate pH 2.5 for terbutaline and with 20 m*M* heptakis(2,6-di-*O*-methyl)-β-CD in 10 m*M* Tris–formic acid pH 3.0 for ephedrine. A sheath liquid flow of methanol–water (50:50 to 90:10, v/v) with or without 2 m*M* ammonium acetate was used at a rate of 2–4 μl/min. The ion spray nebulizing gas was nitrogen at 241 kPa. MS was performed in the positive ion mode. The tentative CE–MS analysis of spiked urine by direct injection with single ion monitoring allowed determination of the analytes with a reported sensitivity 1000-fold better than with a UV absorbance detection.

More recently, Cai and Henion [91] reported the identification and determination of the metabolites of LSD by HPLC and CE with atmospheric pressure ionization–tandem mass spectrometry, but analytical figures of merit were not given.

5. Conclusion

After its relatively recent introduction in the field of analytical toxicology, in the early 1990s, CE has rapidly attracted the interest of enthusiastic researchers, because of its high efficiency, resolution power, economy and, particularly, wide analytical spectrum, including inorganic ions, small drugs and toxicants (with chiral selectivity), small and large biopolymers, metal chelates, etc. Many applications have appeared since then in the leading analytical literature, some of which have been reviewed in the present paper, but surprisingly, forensic toxicology journals, excluding some exceptions, still tend to overlook this new technology. This can be in part attributed to claimed limitations of CE in terms of reproducibility and concentration sensitivity, which with modern

instrumentation and up-to-date analytical methodologies seem to have been almost completely resolved.

In reality, several recently published CE methods report excellent analytical performances, which should allow adoption as a complementary technique to current chromatographic methods and immunoassays.

Also, the particular separation principles that CE relies on make it suitable for confirmation of results obtained by other techniques, particularly when CE is coupled with high information content detection like UV spectroscopy and MS.

Eventually, the possibility of performing different separation modes with a low degree of mutual correlation, by simply changing the separation buffer, without changes in the instrumental hardware, offers additional possibilities of ''internal'' confirmation of results by simply comparing ''orthogonal'' separation techniques (e.g., CZE and MECC) [92].

6. List of abbreviations

AD	Amperometric detection
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
CD	Cyclodextrins
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CGE	Capillary gel electrophoresis
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
CTAB	Trimethylammonium bromide
CZE	Capillary zone electrophoresis
EOF	Electroendoosmotic flow
ESI	Electrospray ionisation
LIF	Laser-induced fluorescence
LLE	Liquid–liquid extraction
MA	Mandelic acid
MAM	6-Monoacethylmorphine
MDA	3,4-Methylenedioxyamphetamine
MDE	3,4-Methylenedioxyethylamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MECC	Micellar electrophoretic capillary chromatography
MS	Mass spectrometry
PGA	Phenylglyoxylic acid
RSD	Relative standard deviations
SPE	Solid-phase extraction
THC	Delta-9-tetrahydrocannabinol
THC-COOH	11-Nor-delta-9-tetrahydrocannabinol-9-carboxylic acid

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