Capillary Electrophoresis Coupled To Mass Spectrometry for Forensic Analysis

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Abstract: In this review the most important techniques, which are developed to hyphenate capillary electrophoresis to mass spectrometry (CE-MS), suitable for forensic analysis, are summarized. Analytes of interest are divided into four main parts, namely, compounds with amine containing side chains, compounds with N-containing saturated ring structures, other heterocycles and peptides. Sample pre-treatments and direct injection modes used in CE-MS for forensic analysis are briefly discussed from critical point of view. Special emphasis is placed to point out the advantages of mass spectrometric detection compared to UV- and laser- induced fluorescence (LIF) detections.

Keywords: Capillary electrophoresis, mass spectrometry, forensic, review, drugs.

1. INTRODUCTION

Electrophoresis methodology is based on the movement of an electrically charged substance under the influence of electric field, and was first described as a separation technique by Tiselius in 1937 [1]. His work, involving the separation of proteins placed between buffer solutions in a tube across an electric field, earned him Nobel Prize for chemistry in 1948 [2]. In early 1980s, Jorgensen and Lukacs [3-5] demonstrated that high performance electrophoretic separations in capillaries were possible. Since the appearance of first commercial instrumentation in 1989, numerous books and reviews have been dedicated to this analytical technique [6-8]. The term capillary electrophoresis (CE) is a family of related techniques that employ narrow-bore (20-200 μm i.d.) capillaries to perform high efficiency separations of both large and small molecules. These separations are facilitated by the use of high voltages (10- 30 kV), which may generate electroosmotic and electrophoretic flow of buffer solutions and ionic species, respectively, within the capillary. The potential of this technique for forensic was first demonstrated in 1991 by Weinberger and Lurie [9]. All techniques of CE, including capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), isotachophoresis (CITP), etc. gained importance as routine method for environmental, pharmaceutical, forensic and biochemical analysis [10]. CE offers high effiency, short analysis time and ability to perform separations in aqueous solutions. Furthermore, the small sample volumes makes CE the analytical tool of choice in applications, which are sample-limited. Capillary based electrophoretic separations offer a number of distinct advantages over their classical counterparts, namely detection capabilities, such as photometry (e.g., UV-photometry) [11], fluorimetry [12], conductivity [13] or electrochemical methods [14]. An additional advantage offers a combined approach of CE and mass spectrometry (MS).

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Electrospray ionisation mass spectrometry (ESI-MS) was invented by Yamashita and Fenn in 1984 [1], which was honoured by the Royal Swedish Academy of Sciences with the Nobel Prize in Chemistry for 2002 partly to John B. Fenn for his pioneering work. Since 1990 ESI-MS has made a significant commercial impact. Along with matrix-assisted laser desorption/ionisation (MALDI) the development of interfaces suitable to couple with CE, has widened the scope of MS analysis to high-molecular-mass compounds such as proteins and nucleotides requiring only a small amount of sample [2]. ESI-MS relies on the production of multiply charged ions whose *m/z* values can be analysed on virtually all types of mass spectrometers (MS). Low-molecular-mass molecules such as drugs and their metabolites have also been subject to investigation by ESI-MS. Detailed structural information on such drugs and their metabolites can be obtained by resorting to cone voltage fragmentation with a single MS instrument [3], collision-induced dissociation (CID) with quadrupole MS instruments, MS^n techniques using quadrupole and time-of-flight (TOF) instruments [4]. ESI has now become the most important ionisation technique not only for the on-line coupling of high-performance liquid chromatography (HPLC) but also for CE [5-7] . CE-ESI-MS and its application to small-molecule analysis have also been reviewed by several other authors. Smyth *et al.* [8], Van Brocke *et al.* [9] have reviewed the progress in hyphenation of CE with ESI-MS in all capillary based electromigration techniques, such as capillary gel electrophoresis (CGE), capillary isotachophoresis (CITP), capillary isoelectric focussing (CIEF), micellar electrokinetic chromatography (MEKC), affinity capillary electrophoresis (ACE), as well as in the hybrid techniques capillary electrochromatography (CEC), and pressurised capillary electrochromatography (pCEC) progress has been made in experimental setups. For many groups of analytes, including peptides, proteins, nucleotides, saccharides, drugs and their metabolites, CE-ESI-MS has been successfully applied. Upon nonaqueous CE and on its interfacing with ESI-MS was reported by Riekkola *et al.* [10]. Plaut and Staub [11] have reviewed the application of CE in forensic toxicology in 2002. They stated that rapid

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analysis, high efficiencies, small sample and reagent requirements, and economical costs were advantages that CE brought to forensic science. On the other hand, lack of sensitivity and maturity meant that CE had not yet been established in forensic laboratories as an additional method, in spite of a very broad spectrum of applications. Huikko *et al.* [12] discussed recent developments in miniaturisation of analytical instruments utilising microfabrication technology. The concept of micrototal analysis systems (μ-TAS), also termed "lab-on-a-chip", and the latest progress in the development of micro-fabricated separation devices and on-chip detection techniques such as MS were discussed. It was stated that also in this case ESI was the interface of choice. Tagliaro *et al.* reviewed chromatographic and electrophoretic/electrokinetic strategies for hair analysis in forensic and biomedical sciences [13]. Other reviews provide a more general overview upon CE procedures using UV- or laser induced fluorescence (LIF) detection in forensic toxicology without mentioning mass spectrometry [28-32].

Search for the keywords "capillary electrophoresis", "mass spectrometry" and "forensic" using the SciFinder database of the American Chemical Society (ACS) results in 107 hits, which are summarized in the present review. The following chapters are divided according to the compounds of main interest into four main parts, namely, analytes with amine containing side chains, analytes with N-containing saturated ring structures, other heterocycles and peptides and include also some methods which have not been setup exclusively for forensic analysis but are highly suitable to it.

2. AMINES

2.1. Amphetamines

Amphetamine was first synthesized in 1887 by the Romanian chemist Lazăr Edeleanu at the University of Berlin, who called it "phenylisopropylamine". Amphetamine is a chiral compound. The racemic mixture can be divided into its optical antipodes: laevo- and dextro-amphetamine. Amphetamine is the parent compound of its own structural class, comprising a broad range of psychoactive derivatives, e.g., MDMA (Ecstasy) and the N-methylated form, methamphetamine. Amphetamine is a homologue of phenethylamine. Traditionally the medical drug came in the racemic salt-form dl – amphetamine sulfate (dl = levo- and dextro-form in equal amounts). In the United States, pharmaceutical products containing solely dl-amphetamine are no longer manufactured. Today, dextro-amphetamine sulphate is the predominant form of the drug used; it consists entirely of the d-isomer. Attention disorders are often treated using Adderall or generic-equivalent formulations of mixed amphetamine salts that contain both d/l-amphetamine and d-amphetamine in the sulfate and saccharate forms mixed to a final ratio of 3 parts d-amphetamine to 1 part l-amphetamine.

CE-MS methods, employing a sheath liquid interface for the analysis of amphetamines, have been reported by Curcuruto *et al.* [33] and Gaus *et al.* [34], but the sheath liquid composition and the electrospray conditions were not optimized in these studies. Therefore, a CE-ESI-MS method, which is suitable for the analysis of ecstasy and related amphetamines in urine samples, was developed by Varesio *et al.* [35]. In this study, the effect of the sheath liquid composition on the sensitivity of the electrospray process was demonstrated. A preliminary evaluation of the coupling parameters was undertaken in order to reach the highest sensitivity of the pneumatically assisted electrospray and to reduce fragmentation, as well as the dilution effect of the electrospray. Boatto *et al.* [19] reported a validated CE-ESI-MS method preceded by an SPE procedure for the screening and determination of ten 2,5-methylenedioxy derivatives of amphetamine and phenylethylamine in human urine, with sensitivity, accuracy and precision comparable to LC-MS techniques. Concerning the sensitivity of the developed analytical method, limit of detection (LOD) and limit of quantitation (LOQ) were found to range from 0.31 to 4.29 ng/mL and from 1.00 to 13.98 ng/mL, respectively. On the other hand, the identification of the investigated amphetamine derivatives in biological samples or confiscated tablets can be facilitated using the obtained mass spectra. Even if the quantitative determination of γ-hydroxybutyric acid, a compound poorly absorbing the UV light, in biological samples has recently been demonstrated in CZE with indirect UV detection [20, 21] , the use of MS, leading to an unambiguous identification, may be necessary for forensic applications. With this aim in view, Gottardo *et al.* [22] developed a CE method using electrospray IT (ion trap) mass spectrometer to achieve the direct and unequivocal detection of this compound in human urine. Despite the absence of sample pretreatment, except a dilution with water (1:4) containing the internal standard, *i.e.* maleic acid, the optimised method was very selective since no interference from the biological matrix or the most common drugs of abuse was observed. Even if the sensitivity of the method (LOD= 20 μg/mL) was sufficient in the case of acute intoxications, it is of limited interest to determine γ-hydroxybutyric acid concentrations in other applications, maybe because of the very poor peak efficiency.

Ramseier *et al.* [23] have carried out confirmatory testing of amphetamines and other designer drugs in human urine by CE-ESI-MS^n. Analytes of interest were amphetamine (A), methamphetamine (MA), 3,4-methylenedioxymethamphetamine (MDMA, Ecstacy) and 3,4-methylenedioxyamphetamine (MDA). For analysis an aqueous pH 4.6 buffer composed of ammonium acetate/acetic acid was applied. Although the effective capillary length employed in the CE-MS setup was longer than in the CE-UV setup, detection time intervals were noted to be significantly shorter and, consequently, resolution was observed to be lower. The presence of a hydrodynamic coflow (siphoning) towards the cathode is presumed to be responsible for that effect. Recording MS/MS data of A, MA, MDMA showed that major fragments are formed due to the loss of NH_2R. Not surprisingly, the major fragments of A and MA were found to be identical. The same was found for MDA and MDMA. The presence of MDMA in urine and its extract was confirmed *via* selected reaction monitoring (SRM). The protonated MDMA ion (m/z 194.1) was isolated with a 2 Th width and subjected to fragmentation with collision energy of 18% maximum. The MDMA concentration in this urine was 3 μg/mL, a concentration that was found to be close to the detection limit when urine was injected directly. The recovery of MDMA was found to be 80%. The analysis of the investigated drugs in a concentration range between 50-200 μg/mL was found to be sufficient for confirmatory testing of most urinary drugs of abuse.

Geiser *et al*. [24] have carried out simultaneous analysis of some amphetamine derivatives in urine by non-aqueous CE coupled to ESI-MS. For the fast baseline separation of six amphetamines, including A, MA, MDA, MDMA, MDEA and MDPA, several ACN-MeOH mixtures containing 25 mM ammonium formate and 1 M formic acid were tested for separation of the investigated drugs. As a result, a ACN-MeOH (80:20, v/v) mixture containing 25 mM ammonium formate and 1 M formic acid was found to be a good compromise in terms of selectivity, high efficiency and short analysis time. Optimisation of sheath-liquid composition showed that applying an isopropanol-water (50:50, v/v) mixture in presence of 0.5% formic acid at an optimised flow-rate of 3 μL min^{-1} gives the best abundance signal as well as the most stable conditions. Optimisation of nebulisation pressure, drying gas flow-rate allowed further improvement of separation efficiency in the non-aqueous mode, whereas it has to be kept in mind that in non-aqueous CE the migration order is inversed compared to the aqueous CE. The authors describe the established system to be very sensitive to slight changes in electrolyte composition, resulting in a significant change in selectivity. For the separation of the two positional isomers MDEA and MBDB which can not be separated applying aqueous CE, 100% ACN was found to be effective. However, as resolution was insufficient for quantitative purposes, the CID approach was selected. The system showed high efficiency (N ~120000) with a relative standard deviation of uncorrected migration time and peak area of 0.5% and 15%, respectively. Improvement was achieved by the addition of an internal standard. The LOD range of this CE method is significantly lower than that estimated by Wu *et al.* [25] in their HPLC method. Finally, analysis of interesting analytes in urine samples was successfully achieved after sample pre-treatment by liquid-liquid extraction (LLE). Lazar *et al*. [43] reported the CE-TOF-MS analysis of small molecules, which contain basic amino functional groups that can be easily protonated in acidic aqueous solutions. A mixture containing amphetamine, methamphetamine, procaine (P) and tetracaine and cocaine was analyzed using CE–TOF-MS at two injection levels, one at 610–850 fmol (Fig. **1a**) and the other much lower (110–135 fmol) (Fig. **1b**). Intense peaks were still observable. It was estimated from observing the background noise and signal intensities for the 110–135 fmol injection that detection limits of approximately 30–50 fmol in the presence of citrate buffer would be obtained (S/N = 3). Selected-ion traces for CE–TOF-MS analysis of drugs of abuse were obtained by integrating ion intensities within narrow *m*/*z* ranges that corresponded to the protonated molecular ions of the analytes. The authors describe a great advantage of TOF-MS where all ions can be monitored simultaneously without loss in sensitivity. Seized samples could easily be analysed by continuous infusion using the micro-electrospray, since the main objective in such analyses is to identify the drug present in the confiscated material. Weak signals corresponding to polymeric contaminants were also present in each of the spectra. The seized sample containing a mixture of amphetamine and methamphetamine was also analysed within 15 minutes using CE–TOF-MS under conditions identical with those used for the previously described separations. The obtained relatively long migration times are caused by the necessity to use a capillary with a minimum length of 85 cm to achieve the interfacing through the liquid sheath source.

Fig. (1). CE–TOF-MS electropherograms of drugs of abuse. Conditions: 85 cm × 50 mm id uncoated fused silica capillary, 25 μm citrate buffer (pH 3), 30 kV, 10 mbar, 7.8 mA; 1 μL min^{-1} CH_3OH–H_2O–CH_3COOH (80 + 20 +0.1 v/v) liquid sheath; ESI (3000 V), 90 °C; MS data acquisition at 5000 Hz, 1000 spectra averaged, 5 data points s^{-1}. A, 30 mbar 3 0.2 min injection (~ 650–810 fmol); B, 10 mbar 3 0.1 min injection (~ 110–135 fmol). Peak identifications: 1, amphetamine; 2, methamphetamine; 3, procaine; 4, tetracaine; 5, cocaine; 6, impurity; 7, heroin. Reprinted from reference [26] with permission.

2.2. Stereoselective Amphetamine Analysis

Sheppard *et al.* [27] performed separations with 5 mM heptakis(2,6-di-*O*-methyl)-β-CD in 5 mM phosphate pH 2.5 for terbutaline and with 20 mM heptakis(2,6-di-*O*-methyl)-β-CD in 10 mM *Tris*-formic acid pH 3.0 for ephedrine. The drug terbutaline (trade names Brethine, Bricanyl, or Brethaire) is a β_2-adrenergic receptor agonist, used as a fast-acting bronchodilator (often used as a short-term asthma treatment) and as a toxolytic to delay premature labour. The inhaled form of terbutaline starts working within 15 minutes and can last up to 6 hours. Ephedrine (EPH) is a sympathomimetic amine similar in structure to the synthetic derivatives amphetamine and methamphetamine. Ephedrine is commonly used as a stimulant, appetite suppressant, concentration aid, decongestant and to treat hypotension associated with regional anaesthesia. Chemically, it is an alkaloid derived from various plants in the genus *Ephedra* (family Ephedraceae). It is most usually marketed in the hydrochloride and sulfate forms. For hyphenation a sheath liquid flow of methanol-water (50:50 to 90:10, v/v) with or without 2 mM ammonium acetate was used at a flow rate of 2-4 μL/min. The ion spray nebulising gas was nitrogen at 2451 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 UV-

absorbance detection, which was clearly demonstrated upon the analysis of a male urine sample containing 27 μg/mL. The same separation was carried out applying selected reaction monitoring (SRM) conditions. For the first time the on-line CE-MS/MS detection and dissociation of a β-cyclodextrin host-guest noncovalent inclusion complex was performed.

Iio *et al.* [28] described a rapid and simple method for the analysis of the d- and l-isomers of seven methamphetamine related compounds and the d-isomer of pseudoephedrine (pseudoEP) with direct injection of urine. The compounds were methamphetamine (MA), amphetamine (AP), dimethylamphetamine, ephedrine, norephedrine, methylephedrine, p-hydroxymethamphetamine (pOHMA). The electrolyte was 1 M formic acid/1 M ammonium formate (10/0.2, v/v) (pH 2.0) containing 1.5 mM heptakis(2,6-diacetyl-6-sulfato)-β-cyclodextrin. The optimised system allowed the baseline separation of 14 enantiomers and d-pseudoEP within 30 min. A urine sample was mixed with an equal volume of internal standard l-1-PEA solution, filtered with a 0.45 μm filter and then injected into the CE-MS system. In an analysis of urine sample from a healthy person spiked with racemic MA and AP, the reproducibility (n = 6) of the migration times and peak areas after correction by an internal standard were under 0.08% and under 3.6%, respectively. The detection limits using selected ion monitoring were 0.01 μg/mL (which corresponds to 0.02 μg/mL urine) for the enantiomers of MA, AP, and pOHMA. The detection yields of the enantiomers of MA, AP and pOHMA from urine were in the range of 97.7-108.8%. Summarising the established method can be described to be rapid, simple, highly sensitive and free from errors and can successfully assay for analytes in urine samples from MA and MDA addicts and patients under selegiline pharmacotherapy.

Cherkaoui *et al*. [29] demonstrated an on-line combination of partial-filling CE and ESI-MS for the enantioseparation of selected drugs and metabolites including amphetamines. A volatile buffer constituted of 20 mM ammonium acetate at pH 4.0, and a polyvinyl alcohol-coated capillary were used for the separation of the chosen model compound methadone. Three different cylcodextrins, namely sulfobutyl ether-β-CD, carboxymethylated-β-CD and hydroxypropyl-β-CD, were selected. The authors applied two different experimental designs: (i) a full-factorial design (FFD) to examine the effects of significance of the investigated factors, and (ii) a central composite face-centered design (CCD) to establish the mathematical model of the selected responses in function of experimental factors including the chiral selector concentration, the separation zone length, and the nebulisation gas pressure. The use of MS in the SIM mode resulted in high selectivity, as well as improved sensitivity compared to UV detection. It was shown, that the chiral selector concentration had a positive effect on enantiomeric resolution, migration time, and apparent sensitivity. The nebulisation gas pressure affected dramatically the quality of the separation. The quality of the mathematical model was further improved by means of a CCD allowing the setup of a robust experimental domain. The method showed good reproducibility in terms of migration times and peak area ratio, leading to an enantiomeric resolution higher than 7.2.

Reversed-polarity (RP) capillary electrophoresis/pos. ion electrospray ionization mass spectrometry (CE-ESI- MS) and tandem mass spectrometry (MS/MS) were utilized by Iwata *et al.* [30] for simultaneous chiral separation of nine amphetamine-type stimulants (ATS) (dl-norephedrine, dl-norpseudoephedrine, dl-ephedrine, dl-pseudoephedrine, dl-amphetamine, dl-methamphetamine, dl-methylenedioxy-amphetamine, dl-methylene-dioxymethamphetamine, and dl-methylenedioxyethylamphetamine). Using highly sulfated γ-cyclodextrin (SU(XIII)-γ-CD) as a chiral selector, the nine ATS were completely separated within 50 min. The migrated ATS-CD complex was dissociated at the ESI interface, and only ATS molecules went into the MS detector so that all 18 individual enantiomers were identified by their mass spectra. The detection limit of MS/MS was 10 times more sensitive than those for single MS. This was the first report that racemic EP and pEP were enantioseparated simulataneously using an MS detector (Fig. **2**). Iwata *et al*. suggested to use the CE-UV method for routine analysis and quantitative analysis, and the CE-MS/MS for confirmation. The detection limit for d-norephedrine was found at 7 pg. The intraday and interday repeatability of the relative migration time (n=5) was than 1.49% and 2.64%, respectively. Finally, the described methodology could support impurity-profiling analysis of seized MA. Schappler *et al.* described the stereoselective analysis of plasma samples containing five amphetamines compounds, namely amphetamine itself, methamphetamine (MA), methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA) and methylenedioxyethylamphetamine (MDEA) [48]. To achieve fast and efficient sample preparation, protein precipitation (PP) and liquid-liquid extraction (LLE) were applied with hydrodynamic and electrokinetic injection. Detection limits of 1 ppm were reached by employing PP and hydrodynamic injection, whereas 1ppb was detected when samples were prepared with LLE followed by electrokinetic injection. For stereoselective determinations in partial-filling mode equivalent detection limits to conventional analysis (0.5 ppb *per* enantiomer) were achieved. Moreover, matrix effect was investigated in CE-ESI/MS with a commercially available coaxial sheath-liquid ESI interface used as postcapillary infusion system. No relevant matrix effect was observed with LLE, while signal suppression occurred out of the analysis migration window by applying PP.

2.3. Methadone

Methadone is a synthetic opioid and was synthesized in Germany in 1937. It is a potent analgesic and sedative, which is used in treatment of heroin addiction. In overdose situations, it causes respiratory depression, hypotension, stupor, circulatory problems and coma. It is a racemic drug whose activity is almost entirely due to the L-isomer, although the D-isomer has analgesic properties in large doses. It is rapidly absorbed after oral administration and is widely distributed in tissues with high concentrations in liver, lung, kidneys, bile and urine. Methadone is metabolised *via* mono- and di-N-demethylation. Methadone and its metabolites have been measured in biological specimens using various procedures including capillary electrophoresis, immunoassay, liquid chromatography/mass spectrometry (LC/MS), EI GC/MS and positive ion GC/MS. Thormannn *et al.* [31] described a competitive binding, electrokinetic capillary-based immunoassay method for urinary methadone analysis. After incubation of 25 mL urine with the reactants, a small aliquot of

Fig. (2). MS/MS spectra and expected fragmentation of ATS. Reprinted from reference [30], with permission.

the mixture was applied onto a fused-silica capillary and the unbound fluorescein-labeled methadone tracer was monitored by capillary electrophoresis with on-column laser-induced fluorescence detection. Configurations in presence and absence of micelles were investigated, capable of recognizing urinary methadone concentrations $\geq$ 10 ng/mL, and suitable for rapid methadone screening of patient urines. Based upon shorter run times and a much better separation of free tracer and antibody-tracer complex, conditions without micelles were preferred. For confirmation analysis of urinary methadone and its major metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), capillary electrophoresis in a pH 4.6 ammonium acetate-acetic acid buffer was inter-phased to an atmospheric pressure ionisation (APCI) triple quadrupole mass spectrometry system. Using positive ion electrospray ionisation and the tandem mass spectrometry mode with collision-induced dissociation (CID) in the collision cell, fragmentation of the 2 substances was detected. For confirmation *via* direct urine injection or application of a urinary extract, in-source fragmentation was employed and the 1st quadrupole was operated in the selected ion monitoring mode (SIM) by switching between the masses of relevant precursor/product ion sets for methadone (m/z = 310, 265) and EDDP (m/z = 278, 249, 234). This capillary electrophoresis-mass spectrometry approach was shown to permit the confirmation of methadone and EDDP in patient urines that tested positive for methadone using electrokinetic capillary-based immunoassays, a fluorescence

polarisation immunoassay, and capillary electrophoresis with UV absorption detection.

Rudaz *et al.* [32] have demonstrated that the partial-filling technique is appropriate for the chiral separation of chiral basic drugs such as MTD. An anionic carboxymethyl β-cyclodextrin (CMB) was applied as a chiral selector in a PVA coated capillary as well as in a conventional fused-silica capillary. MTD enantiomers were separated using 40 mM ammonium acetate buffer, pH 4.0, containing 1 mg mL^{-1} CMB. Under these electrophoretic conditions the analysis of methadone enantiomers in presence of other metabolites could be performed without ambiguity. Moreover, in comparison to UV detection, working in the SIM mode increased sensitivity by a factor of 10, allowing the monitoring of MTD in serum samples. Finally, the optimised method was applied to serum samples of patients undergoing methadone therapy. The optimised system was also used for the analysis of venlafaxine, a second-generation antidepressant drug, and its metabolites as well as for tropane alkaloids.

2.4. Clenbuterol and Salbutamol

Clenbuterol and salbutamol are kinds of β-agonists, which are originally developed for the treatment of chronic obstructive pulmonary diseases, especially for asthma. Additionally, these adrenergic drugs use meat-producing industry by enhancing muscle accretion and diminishing muscle protein breakdown. However, the use of potent illegal β-agonists continues to demand regulatory scrutiny since they can cause cardiovascular and other side effects to human. Consequently, analysis of β-agonists is essential for both quality control of drugs in human uses and for monitoring of illegal uses in meat industry. Different methods, HPLC, GC, CE and hyphenated techniques, have been proposed for the analysis of various β-agonists in pharmaceuticals and biological fluids and animal tissues. The coupling of in-capillary isotachophoresis (ITP) with CE-MS was proposed by Lamoree *et al.* [33] 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 mM ammonium acetate pH 4.8-methanol (1:4, v/v), while the terminating buffer was composed of 50 mM β-alanine pH 4.8-methanol (1:4, v/v). Samples were dissolved in CE buffer and hydrodynamically injected into the capillarywhich was filled with the 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 CE separation. A single-quadrupole MS equipped with an electrospray interface was used in the positive ion mode.

Toussaint *et al.* [34] have investigated the on-line coupling of partial filling-CE with MS for the separation of clenbuterol enantiomers. The background electrolyte was a mixture of 10 mM ammonium acetate buffer adjusted to pH 2.5 with acetic acid and methanol (80:20 v:v) containing 40 mM DMCD. The samples were injected by hydrodynamic injection at 20 mbar for 0.2 s (5 nL injected). The separation was achieved at 20°C by applying +30 kV and +10 mbar at the capillary inlet. The sheath liquid was of the same composition as the background electrolyte and flow rate was set to 3 μL/min. The mass spectrometer was set to generate and to select positive pseudomolecular ions at mass-to-charge ratio, m/z, 277.1 for (R,S9-clenbutrol and 239.9 for (R,S)-salbutamol. The MS/MS fragmentation of the pseudo molecular ion was achieved with a collision energy of 16 eV. Fragment ions at m/z 202.8 and m/z 147.7 for clenbuterol and salbutamol, respectively, were subsequently selected for all further analyses employing MRM detection. For the analysis of these analytes in plasma samples, solid-phase extraction (SPE) was employed prior to injection onto the CE-system. By this method, clenbuterol enantiomers were successfully resolved and separated from salbutamol (internal standard) in aqueous solution and in plasma samples. Mazereeuw *et al.* [35] have used on-line isotachophoretic sample focusing for loadability enhancement in CEC-MS. As a stationary phase a silica-C18 packed capillary column with retaining frits was employed. A coupled-column ITP-CEC setup was used with a 220 μm inner diameter capillary, in which counterflow focusing was performed, connected *via* a T-junction to a 75 μm ID. CEC capillary. It was shown upon the injection of neostigmine, salbutamol and fenoterol in the nmol/l range that a signal enhancement of a factor 1000 can be achieved, while the ITP-CEC separation efficiencies are between 120 000 and 140 000 plates/m. An ITP-CEC analysis of a 15 μL urine sample spiked with 3.3 μmol/l neostigmine and salbutamol and 1.6 μmol/l fenoterol was recorded after desalting the sample applying SPE. Due to the high amount of matrix ions, the ITP focusing time was extended significantly and could take up to 40 minutes. Servais *et al.* reported the on-line coupling of cyclodextrin mediated nonaqueous capillary electrophoreses (NACE) with ESI-MS for the mass spectrometric determination of salbutamol enantiomers in human urine preceded by solid-phase extraction as sample cleanup [54]. After optimisation of several parameters, a limit of quantification of 18 and 20 ng/ml was obtained for salbutamol enantiomers. This method can be considered as a powerful technique which provides an enantioselective bioanalytical tool for detecting and quantifying enantiomers of drugs *in vivo*.

Loden *et al.* [36] have developed a nonaqueous CE method using the partial filling technique with UV and MS detection for the separation and detection of enantiomers. The chiral selector (-)-2,3:4,6-di-O-isopropylidene-2-keto-L-gluconic acid ((-)-DIKGA) has a rigid structure and possesses hydrogen accepting ether functions in the vicinity of the carboxyl group. Different solvent mixtures were studied, as well as different concentrations of (-)-DIKGA and ammonium acetate in the background electrolyte. A partial filling technique was developed with a 39 cm selector plug corresponding to 65% partial-filling composed of (-)-DIKGA and ammonium acetate in a solvent mixture of methanol and 2-propanol. The developed partial-filling system was coupled to a Q-Tof mass spectrometer using a sheath-liquid electrospray ionisation interface. The separated enantiomers were

detected by a TOF-MS system equipped with a sheath-flow ESI interface.

3. N-CONTAINING RING STRUCTURES

3.1. Morphine, Codeine

Morphine and codeine are opioids that are widely used therapeutically and consumed illegally. Therefore their identification in biological samples is important for clinical and forensic toxicology, in pharmacodynamic, pharmacokinetic and pharmacogentic research. Capillary electrophoresis was used for separation and determination of morphinane alkaloids in various matrices—in pharmaceutical formulations, in opium, bound as glucuronides in body fluids, or in samples of forensic interest. Wey and Thormann. [37] used an aqueous background electrolyte consisting of 25 mM ammonium acetate and NH_3 (pH 9.0) for the CE-ESI-MS^n analysis in the positive ion mode of morphine, codeine and related compounds in urine. In the search for a suitable buffer, an aqueous standard solution containing OCOD and OMOR (10 μg/mL each) was analysed in the P/ACE using a 50 μm I.D. capillary of 87/80 cm total/effective length and buffers composed of 25 mM ammonium acetate solution that were adjusted to pH 7.8, 8.3, 8.8, 9 and 9.3 with 1 M NH_3. At pH 7.8 the two analytes were note baseline resolved, and at pH 9.3 there was only one peak. At the three other buffer values the resolution was good. Best results were obtained at pH 9.0. Reference mass spectra of oxycodone (OCOD), oxymorphone (OMOR), noroxycodone (NOCOD) and noroxymorphone (NOMOR), which are opoids that carry an OH group at position 14, were analyzed *via* syringe inlet using aqueous standard solutions of 10 μg/mL and together with MS^2 and MS^3 data they were stored in a computer library. It was interesting to note that all tested compounds lost H_2O at the MS^2 level, i.e. after isolation and fragmentation of the [M+H] ions. This neutral loss seems to be a characteristic behaviour for opioid structures carrying an OH group at position 14 of the molecule. The same was found for the structurally related antagonist naloxone. The detection limits (S/N=3) after hydrodynamic injection (76 mbar for 18 s) of urinary liquid-liquid extracts were found to be about 10 ng/mL (spiking level) for OCOD and OCOD3, 50 ng/mL for NOCOD, NOCOD3, OMOR and OMORd3 and about 300 ng/mL for NOMOR. After electrkinetic injection these values could be slightly further improved. The reproducibility of the signal intensities was found between 4.45% and 25.6% for all analytes. Finally, the optimized procedure was applied for the analysis in urinary samples. For the monitoring of OMOR it was necessary to treat urine with β-glucuronidase/arylsulfatase.

Wey and Thormann [57] described their head-column field-amplified sample stacking (FASS) in presence of a water plug inserted at the capillary tip to be a robust approach providing more than 1000-fold sensitivity enhancement when applied to low-conductivity samples. Electroinjection allowed the determination with ng/mL sensitivity. Urinary samples were twofold diluted prior to analysis by CE-ion trap MS^2 and MS^3, sample pre-treatment was carried out by SPE and LLE. With electroinjection from diluted urine and urinary SPE-extracts, the presence of free opioids and their glucuronic acid conjugates could be unambiguously confirmed in urines that were collected after single-dose administration of small amounts of opioids (7 mg codeine and 35 mg dehydrocodeine, respectively).

Jussila *et al.* [39] reported on the use of a modified liquid junction interface for nonaqueous CE-MS. In their approach a very thin glass spray capillary (30 μm O.D.) was partly inserted inside the CE capillary, the junction was surrounded by the electrolyte medium, which was in contact with the platinum electrode. Testing the interface with five pharmaceuticals methadone, pentazocine, levorphanol, dihydrocodeine and morphine resulted in detection limits ranging from 12 to 540 fmol. It is worthy to note, that the reproducibility of the method was influenced by variations in the spray needle position with respect to the MS inlet.

A rapid, sensitive method for the determination of morphine and amphetamine using capillary zone electrophoresis coupled with electrospray interface (ESI), ion-trap tandem mass spectrometry (CE-ESI-MS^2) was developed by Tsai *et al.* [40]. Morphine and amphetamine were separated in 20 mM ammonium acetate buffer (pH 6.6) and detected by ion-trap mass detector in different analytical time segments (0-6.25 min for amphetamine and 6.25-12.0 min for morphine) in which the tune file for each compound was used separately. Molelucar ions of morphine (*m/z* 286) and amphetamine (*m/z* 136) were detected at 5.77 and 6.83 min, respectively, while product ions of MS^2 for each compound (*m/z* 229, 201 for morphine and *m/z* 119 for amphetamine) were detected almost exactly at the same time with their parent compounds. The limits of detection (LOD) for MS^2 determination were 30 and 50 ng/mL for amphetamine and morphine, respectively, with an S/N ratio of 3. For more sensitive detection of morphine, the sample was injected for a longer time (i.e., 80 s) and hydrodynamically transported into a CE capillary for MS detection. Morphine and its product ion appear at 0.36 and 0.39 min on the ion chromatogram, respectively, with a five-fold increase of detection sensitivity (LOD, 10 ng/mL). The CE-MS system thus established was further applied for forensic urine samples screened as morphine-positive by fluorescence polarisation immunoassay (FPIA) (Fig. **3**). These results indicated the feasibility of CE-ESI-MS^2 for confirmative testing of morphine in urine sample.

3.2. Alkaloids

The family of alkaloids is generally characterized by the presence of heterocyclic bound nitrogen, the basic reactivity and physiological effect on humans and animals. Especially because of strong pharmacological effects substance group is of special interest. Many of them are widely used as medicines for treatment of illness (atropine, codeine) and cancer (vincristine). Originally, alkaloids were thought to be unique in plant kingdom, but in recent times, however, they have been detected in some animals, e.g. in the toxic secretions of fire ants, ladybirds and toads. Classification is usually based on type of ring system present and on biosynthetic origins. Alkaloids are detected in plant extracts by number of well-known colour tests, e.g. general tests like Dragendorff and Mayer and/or specific ones such as Ehrlich. Beside chromatographic separation and analysis methods like thin layer chromatography (TLC), GC, HPLC and combined techniques like GC-MS or HPLC-MS, capillary electrophoresis is a frequently used separation technique. The hyphenation

Fig. (3). Ion-chromatogram of forensic urinary morphine with concentrations of (a) 0.45 and (b) 5 μg/mL. The samples were injected for 40 seconds and separated by CE with a voltage of 20 kV for 10 minutes. The parent ion (*m/z* 286) was monitored in full scan mode within a 50-350 mass range while the product ions (*m/z* 229, 201) were selected on the MS^2 filter of the parent ion. Reprinted from reference [40], with permission.

of CE and MS is an attractive combination of two analytical techniques with the potential of high-resolution from CE and excellent sensitivity from MS. Huhn *et al*. [41] described the simultaneous hyphenation of capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection and electrospray ionization-mass spectrometry (ESI-MS) as a novel combined detection system for CE. β-Carbolines were chosen as model analytes with a forensic background. β-Carbolines, derived from *Banisteriopsis caapi* and *Psychotria viridis*, are the active ingredients of ayahuasca, the famous psychotropic drink and sacramental drug of the Shamans from the Amazon and Orinoco river basins. Nonaqueous CE as well as conventional CE with an aqueous buffer system is compared concerning efficiency and obtainable detection limits. The distance between the optical detection window and the sprayer tip was minimised by placing the optical cell directly in front of the electrospray interface. Similar separation efficiencies for both detection modes could thus be obtained. No significant peak-broadening induced by the MS interface was observed. The high fluorescence quantum yield and the high proton affinity of the model analytes investigated resulted in limits of detection in the femto-gram (nmol/L) range for both detection methods. The analysis of confiscated ayahuasca samples and ethanolic plant extracts revealed complementary selectivities for LIF and MS detection. Thus, it is possible to improve peak identification of the solutes investigated by the use of these two detection principles.

Reddy *et al*. [42] reported on the application of capillary zone electrophoresis in the separation and determination of the principal gum opium alkaloids i.e., thebaine, codeine, morphine, papaverine and narcotine. The separation was carried out by using a 7:3 mixture of methanol and sodium acetate (100 mM, pH 3.1) at a potential of 15 kV. The influences of buffer composition, pH and voltage on the separation of alkaloids was studied in detail. The detection limit of each alkaloid dissolved in methanol was found to be 850 ng/mL (morphine), 450 ng/mL (thebaine), 500 ng/mL (codeine), 550 ng/mL (papaverine), and 500 ng/mL (narcotine) with a signal to noise ratio of 3:1. Percentage compositions (g%) of opium alkaloids in five gum opium samples were found to be in the range of 14.45-15.95 (morphine), 2.0-3.45 (codeine), 1.32-2.73 (thebaine), 0.92-2.37 (papaverine), and 3.85-5.77 (narcotine).

4. OTHER HETEROCYCLES

4.1. Zaleplon

Zaleplon a pyrazolopyrimidine, is a sedative and hypnotic agents with a chemical structure unrelated to benzodiazepines. Zaleplons have several side-effects (e.g. visual disturbance, hallucinations, hypotension), including abuse potential, interactions with other CNS depressants, impairment of psychomotor performance and memory and risk of overdose. At present, immunoassays dedicated to the detection of zaleplon in biofluids are not yet commercially available; therefore their detection relies only on chromatographic procedures. Several analytical methods, using HPLC with fluorescence or diode-array detection and CE with laser-induced fluorescence detection have been already described for the determination of Zaleplon. A method for the separation and identification of zaleplon metabolites (Fig. **4**) in human urine using CE with laser-induced fluorescence detection and LC-MS was described by Horstkötter *et al*. [62]. Carboxymethyl-β-CD was used as a charged carrier. LC-MS was employed for the identification of metabolites,which were 5-oxozaleplon, 5-oxo-N-deethylzaleplon and 5-oxozaleplon glucuronide. N-Deethylzaleplon as well as zaleplon itself could not be detected in human urine by the CE-LIF assay. Limits of quantification by the CE-LIF assay including a ten-fold preconcentration step by SPE were 10 ng/mL for zaleplon and N-deethylzaleplon and 100 ng/mL for 5-oxozaleplon and 5-oxo-N-deethylzaleplon.

5. PROTEINS

Peptides can play a role in forensic science as blood doping agents. The most prominent erythropoietin (EPO) is a glycoprotein hormone that is a cytokine for erythrocyte (red blood cell) precursors in the bone marrow. The standard

Fig. (4). Metabolic pathways of the phase I metabolism of zaleplon.

method of testing recombinant human erythropoietin (rhEPO) is a combination of blood and urine test. The blood test is navigated primarily through the variation in blood parameters, whereas the urine test includes an immunoblotting assay based on isoelectric focusing separation step, two blotting steps and luminescence detection. So whole practice is quite time-consuming and complex. Yu *et al.* [44] reviewed the separation and detection of erythropoietin by CE and CE-MS. They mention as the main drawback of UV-detection its relatively low sensitivity (10^{-5}–10^{-6} M). Comparatively, the on-line coupling of CE with MS is an attractive technique for the analysis of erythropoietin, because MS detection has the advantage over both UV–Vis and LIF detection [45]. Nevertheless, detection of rhEPO by the on-line CE–ESI-MS technology is still subject to various difficulties, including the concentration of CE buffer, the type, composition and flow-rate of sheath liquid, and the temperature and flow rate of drying gas. The effects of these factors on the CE–ESI-MS detection need to be studied systematically. Compared to the intact molecule, the digested rhEPO fragments are easier to be detected by on-line CE–ESI-MS method. Zhou *et al.* [46] have characterized 17 tryptic fragments in the total of 21 non-glycosylated and glycosylated peptides by the CE–ESI-MS method in order to study the amino acid sequence of rhEPO. It has been shown that CE–ESI-MS is a powerful technique for peptide-mapping analysis. The results demonstrated that, when the internal walls of the capillaries were permanently coated with 6,6-ionene and a volatile acetate buffer was used with a mixed solvent of water/acetonitrile containing 5 mM ammonium acetate as the sheath liquid, not only the on-line MS ion signal of uEPO was achieved, but also baseline separation of three major rhEPO glycoforms was successfully and reproducibly obtained. Furthermore, the mixture of rhEPO and uEPO was separated, resulting in two incompletely resolved peaks (Fig. **5**) that were identified to be rhEPO and uEPO by the unique MS ''fingerprint'' [47]. It can be concluded that, compared with other indirect methods, the on-line CE–ESI-MS technique, combining the advantages of both CE and MS, shows great potential for the direct separation and detection of rhEPO used for doping in competitive sports.

Since the glycosylation pattern of rhEPO depends, among other things, of the cell line used for production, it presents a glycoform scheme different from manufacturer to manufacturer and different also from endogenous rhEPO. The separation of these different glycoforms is interesting since it could be useful: (i) for determining the glycoform distribution in recombinant products from different batches and/or manufacturers, (ii) for studying the link between glycosylated forms and their activity, and (iii) for helping to discriminate between endogenous and recombinant EPO. Lopez-Soto-Yarritu *et al*. [48] describe the development of three different capillary electrophoresis methods that use UV detection to separate EPO glycoforms. The special features of the three methods are discussed in terms of resolution of bands of rhEPO glycoforms, method reproducibility, and compatibility with other more sensitive detection systems such as laser induced fluorescence using on-column derivatisation protocols or more selective ones such as mass-spectrometry.

Sanz-Nebot *et al*. carried out the separation and characterisation of recombinant human erythropoietin (EPO) glycoforms by employing CE-ESI-MS and MALDI/TOF-MS, using volatile electrolytes [68]. They are separated according to their charge-to-mass ratios, primarliy due to the differences in their sialic acid content. Various capillary coatings, working conditions, pH variation of buffers were investigated. A conducive separation was achieved when internal walls of the capillaries were coated with polybrene and buffer electrolyte contained 400 mM of acetic acid-ammonium acetate, pH 4.75. Upon detection, MALDI/TOF-MS provided good characterisation of average molecular mass of EPO entity. They are extensively misused as performance enhancing agents in sports. The glycoforms and their quantitative amounts facilitate to recognize the types of EPO hormone whether they are naturally or artificially generated.

A detailed review upon the CE-MS analysis of doping substances was provided by Szokan *et al*. [49] and Bing Yu *et al*. [70]. Special emphasis is put on stimulants, β-blockers, diuretics, exogenous and endogenous steroids and peptide hormones. The efficiency of CE-MS compared to LC-MS and GC-MS is evaluated and discussed.

An overview upon most important CE-MS methods in forensic analysis is provided in Table **1**. Capillary electrophoresis coupled mass spectrometry has started to overpass traditionally used detection techniques, especially CE-UV,

Fig. (5). CE-ESI-MS separation of a mixture of rhEPO and uEPO by using 6,6-ionene-coated capillary. Separation conditions: applied voltage, -10 kV; capillary temperature: 25°C; capillary: a fused silica capillary dynamically coated with PB with 98-cm total length (47 cm effective length) and 50 μm ID; sample: mixture of the 1.0 mg/mL rHuEPO and 20 units/lL uEPO (1:1, v/v); injection: 50 mbar for 20 s; separation buffer: 10 mM of HAc/NH_4Ac at pH 4.75; UV detection at 214 nm; sheath liquid: 5 mM NH_4Ac in MeCN:H_2O (1:1 v/v) at 10^{-1} L/min. Reprinted from reference [47], with permission.

Table 1. Overview upon most important CE-MS methods in forensic analysis

Compound	Matrix	Remark	Reference
2,5-methylenedioxy amphetamines	urine	LOD 0.31 to 4.29 ng/mL	[19]
γ-hydroxybutyric acid	urine	LOD= 20 μg/mL	[22]
Amphetamines	urine	CE-ESI-MS^2	[23]
Amphetamines	urine	non-aqueous CE coupled to ESI-MS	[24]
Amphetamines	urine	CE-TOF-MS	[26]
Amphetamine	spiked urine	stereoselective analysis	[27]
Metamphetamines	urine	stereoselective analysis	[28]
Amphetamines, methadone	serum	stereoselective analysis	[32]
Amphetamine-type stimulants (ATS)	urine	stereoselective analysis	[30]
Methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	urine	fluorescein-labeled methadone	[31]
Methadone	urine	chiral analysis	[32]
Clenbuterol and salbutamol	calf urine	isotachophoresis	[33]
Clenbuterol enantiomers, Salbutamol	plasma	chiral analysis	[34]
Salbutamol	plasma, urine	CEC-MS	[35]
Salbutamol	standards	nonaqueous CE	[36]
Morphine, codeine	urine	CE-ESI-MS^n	[37]
Opioids	urine	head-column field-amplified sample stacking (FASS)	[38]
Oxycodone, metabolites	urine	CE-MS^n	[39]
Morphine and amphetamine	urine	CE-MS^n	[40]
β-Carbolines	ayahuasca samples	nonaqueous CE	[41]
Opium alkaloids	urine, serum	routine analysis	[42]
Zaleplon	urine	CE-LIF	[43]
Erythropoietin	urine, serum	doping	[44]
Erythropoietin glycoforms	urine, serum	doping	[48]

whereas many CE procedures have not been coupled to mass spectrometry yet. In the future CE-MS techniques applied in forensic analysis might help to identify even traces of lower concentrated analytes. After validation, CE-MS will definitely constitute a real alternative to LC-MS for routine quantitative analysis.

6. CONCLUSIONS

The present survey shows the range of possible applications of CE separations coupled on-line to MS in forensic science. Thereby, the article is divided into four main chapters according to the analytes mostly important in forensic science: Analytes with amine containing side chains, analytes with N-containing saturated ring structures, other heterocycles and peptides. This summary should not only help to provide a fast overview upon the discussed topic but also function as a basis for further development of CE-MS methods in forensic analysis.

ABBREVIATIONS

ACE = Affinity capillary electrophoresis

AP = Amphetamine

APCI = Atmospheric pressure chemical ionisation

ATS = Amphetamine-type stimulants

CCD = Central composite face-centered design

CD = Cyclodextrine

CGE = Capillary gel electrophoresis

CIEF = Capillary isoelectric focussing

CEC = Capillary electrochroamtography

CZE = Capillary zone electrophoresis

(-)-DIKGA = (-)-2,3:4,6-di-O-isopropylidene-2-keto-L-gluconic acid

EDDP = 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
FASS = Field-amplified sample stacking
(FFD) = Full-factorial design
FPIA = Fluorescence polarization immunoassay
HPLC = High-performance liquid chromatography
ITP = Isotachophoresis
LC = Liquid chromatography
LIF = Laser induced fluorescence
LLE = Liquid liquid extraction
MA = Methamphetamine
MS = Mass spectrometry
MTD = Methadone
NOCOD = Noroxycodone
NOMOR = Noroxymorphone
OCOD = Oxycodone
6OCOL = 6oxycodol
OMOR = Oxymorphone
pCEC = Pressurised capillary electropchromatography
pOHMA = p-hydroxymethamphetamine
pseudoEP = Pseudoephedrine
rhEPO = Recombinant human erythropoietin
SPE = Solid-phase extraction
TOF = Time of flight
uEPO = Human urinary erythropoietin
μ-TAS = Micrototal analysis systems
ZAL = Zaleplon

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