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1. INTRODUCTION AND AIMS OF THE THESIS

Forensic science is a peculiar scientific discipline that includes all those scientific technologies and applications that are valuable to support the forensic investigation and/or to bring scientific evidence to court. On many occasions, forensic science provides also the information needed for drafting new laws and rules requiring scientific knowledge [1-3]. The main areas of this discipline are forensic toxicology, analysis of explosives, gunshot residues and chemical warfare, trace analysis (fibres, hairs, inks, dyes, glass etc.), and forensic genetics. For the latter area, which is based on DNA fragment analysis and sequencing, the development of capillary electrophoresis (CE) technology has been a fundamental step forward [4], being today a standard technique worldwide.

In almost all its fields of application, the development of analytical forensic sciences finds two milestones: *i.* the introduction of chromatography and electrophoresis and *ii.*, more recently, the introduction of mass spectrometric techniques. In both chromatography and electrophoresis, the miniaturisation of the separation compartment in a capillary format has highly increased the analytical efficiency and improved the possibility of coupling with mass spectrometry (MS).

In the recent decades, CE proved to be a powerful analytical technique because of its excellent separation efficiency, high resolution and selectivity, short analysis time, low reagent consumption, sample volume and cost, and good recovery [5]. In particular, when performing enantioselective analyses, CE is known for being one of the most favoured separation techniques due to its numerous advantages listed above [6].

On these grounds, the major aims of the present doctoral research project were *i.* to improve and to develop analytical methods in CE for the stereoselective separations of NPSs (New Psychoactive Substances) and of drugs involved in dependence withdrawal therapy; *ii.* to couple CE with MS in order to improve analytical sensitivity and selectivity.

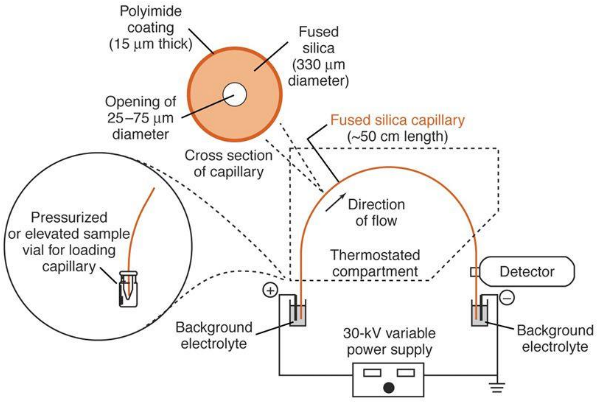
With regards to *i.*, a CZE (Capillary Zone Electrophoresis) method was developed for the chiral separation of ketamine and its major metabolite, norketamine, in hair specimens, in order to investigate potential chronic abuses in the context of traffic and workplace law enforcement. Moreover, an existing analytical method for the measurement of baclofen was further improved, being baclofen a drug that proved effective when administered to alcohol dependent patients in order to reduce the severe symptoms of sudden withdrawal from alcohol abuse.

Last-but-not-least, CE, originally equipped with a UV detector, was coupled to a TOF (Time Of Flight) mass spectrometer with the purpose of taking advantage from the unique features of this mass spectrometry technique, i.e. possibility of acquisition of the "total ion current" without sacrificing sensitivity, possibility of re-processing of the raw data for post-analysis search of compounds, high speed of data acquisition. Moreover, since forensic toxicology involves complex biological matrices, the matrix effect was investigated with the aim of evaluating the possible advantages on ion suppression offered by a miniaturised separation technique, such as CE.

2. CAPILLARY ELECTROPHORESIS

2.1 BACKGROUND

The origin of CE may be considered as the upgrading of an existing technique, namely electrophoresis: they share the same basic principles but differ from each other in the physical compartment where the electrophoretic separation takes place. Electrophoresis is based on the migration of charged particles in a solution under the influence of an electric field. Different particles with different effective charges and/or sizes migrate with different velocities: this is the basic principle of all electrophoretic separation methods. While traditional electrophoresis is applied to slab gels, in CE the separation compartment is a narrow capillary filled with an electrolyte solution. The electric field is applied with an external voltage source between two electrodes in small vials in contact with the solution at both ends of a separation compartment (Figure 1). The narrow capillary is generally made of fused silica and is enclosed in a thin layer of polyamide. The surrounding polyamide acts like a "shell" that protects the glass capillary which is very fragile also because of its minute dimensions (the most commonly used capillaries have an inner diameter between 25 and 100 µm). However, an appropriate portion of the polyamide needs to be removed (burned and cleaned in manually prepared capillaries) in order to create the so-called *detection window*, i.e. where the detection occurs.



D. C. Harris, *Quantitative Chemical Analysis*, 6th ed., 2003, p. 655.

Figure 1. Apparatus for capillary electrophoresis. One way to inject the sample is to place the capillary in a sample vial and apply pressure to the vial or suction at the outlet of the capillary [7].

Since its institution in the early 1980s, CE played a crucial role as a separative technique in selected analytical fields, from biopharmaceutical to environmental studies, and from biomedical to forensic sciences. Such variety of fields of applications lies in the key benefits of this peculiar technique:

- Minimal consumption of sample amounts (in the range of nanolitres), which is extremely important when dealing with exceptionally low quantities of and/or degraded samples;
- Extremely low consumption of solvents (in the order of millilitres compared to litres of the commonly used liquid chromatography), resultant into much lower production of waste to be discarded;
- Wide application window with regards to the analysed compounds (from inorganic ions to DNA fragments, from proteins to entire viruses), (Figure 2);
- Wide variety of separation modes (which will be listed and briefly discussed in section 2.4);

- Diversity of detection systems, allowing the coupling with both UV and fluorescence detection, as well as with MS;
- Extremely high resolution power and separation efficiency;
- Inexpensive maintenance, especially considering the low costs of capillaries against those of chromatographic columns;
- Method development is relatively rapid and the same method (or slightly modified methods) may be applied to various separation analyses.

Thanks to most of the listed advantages and being based on specific separation mechanisms, CE plays a prominent role as an independent analytical technique complementary to chromatography [8].

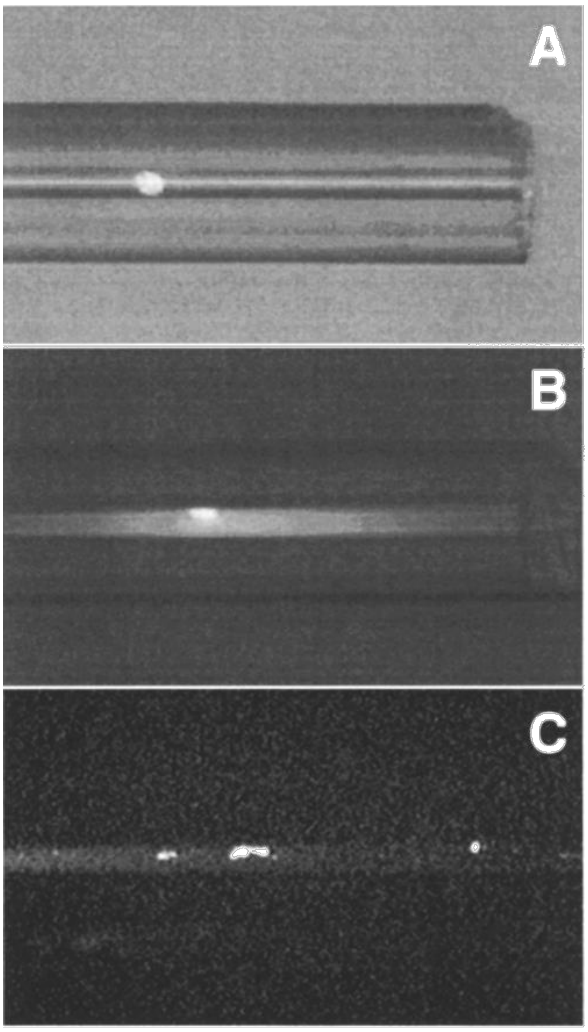


Figure 2. Micrographs showing (a) fluorescent image of a single, intact cell immediately after injection into the capillary and (b) 30 s after injection. In (c), cellular debris begin to separate 10 s after application of high voltage [9].

Interestingly enough, such features provided CE with the recognition of being admissible in the US courts, already in 1996, as a method of evidence. Kuffner et al. used CE to explain how new science may meet the requirements of the federal rules of evidence and those defined by the so-called Daubert Standard, still adopted in the US courts [10].

On a contradictory note, despite such numerous positive aspects, it appears as if CE received poor interest in application in the forensic field in the last decades although it remains a standard technique in forensic DNA analysis and profiling.

For this reason, the purpose of the following paragraphs is to elucidate the basic principles of CE by giving a description of the different modes of separation and detection, and by emphasising its significance and relevance as a separative technique.

2.2 BASIC PRINCIPLES

As mentioned above, CE is a high-voltage electrophoresis occurring in a narrow separation compartment, which offers minimal band diffusion and improved joule heating control, hence allowing the employment of high voltages (10-30 kV). Moreover, the reduced zone broadening results in remarkable separation efficiency values up to 10^{16} theoretical plates.

Migration of charged particles occurs thanks to the phenomenon in which the electrostatic force exerted on a particle is proportional to the neat charge of the particle q_i and the electric field strength or voltage gradient E in the solution according to:

$$F = E \cdot q_i$$

The direction of the force is obviously towards the electrode with a charge opposite to that of the charged particle. This is accelerated and starts migrating. Its migration is then opposed by viscous forces in the solution, which increase proportionally with the velocity v of the particle. After a very short acceleration time, the opposing forces (electrostatic and viscous) cancel each other out and the particle then moves with a constant velocity. Such velocity depends on their electrophoretic mobility μ, which is specific for each individual ionic species according to the mass-to-charge ratio and is described by the following equation:

$$\mu_i = q_i/6\pi\eta r_i$$

where μ_i = ion mobility

q_i = ion charge

η = electrolyte solution viscosity

r_i = ion effective radius.

Since the ion mobility has also been defined as:

$$\mu_i = v_i / E$$

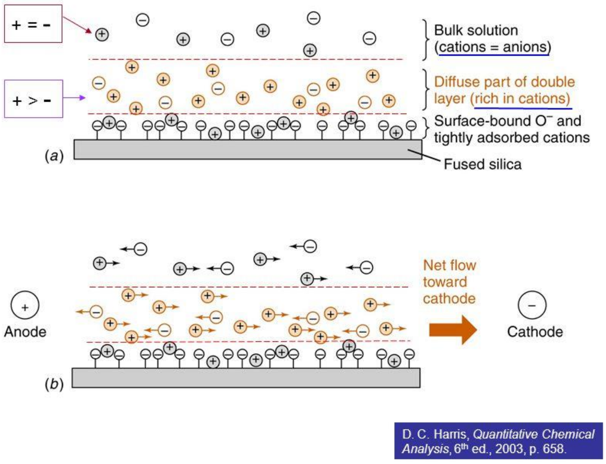
It can be derived that when an electric field of strength E is applied, the ion migration velocity v_i will equal the product $\mu_i \cdot E$, causing the physical separation of the components of a mixture of molecules with different ion mobilities:

$$v_i = \mu_i \cdot E$$

where the electric field strength will be influenced by the applied voltage V and the distance between electrodes d according to the following equation:

$$E = V/d$$

Another peculiar phenomenon that arises inside the separation compartment is known as *electroosmosis*. The inside wall of a fused-silica capillary is covered with silanol groups (Si-OH) with a negative charge ($Si\text{-}O^-$) above pH ~ 2. When an aqueous solution of electrolytes, such as the running buffer employed for the separation, comes into contact with the wall of the capillary, a charge difference between the wall and the solution occurs. Figure 3 shows the *electric double layer* at the wall of the capillary. The double layer consists of fixed negative charge on the wall and excess cations near the wall. Since, under most experimental conditions, the wall is negatively charged and the system is electrically neutral as a whole, the aqueous solution of electrolytes has a net positive charge.



D. C. Harris, *Quantitative Chemical Analysis*, 6th ed., 2003, p. 658.

Figure 3. (a) Electric double layer created by negatively charged silica surface and nearby cations. (b) Predominance of cations in diffuse part of the double layer produces net electroosmotic flow toward the cathode when an external field is applied [7].

This excess of positive ions will allocate in the solution close to the wall, because of the electrostatic attraction by the negative wall. When a voltage difference is applied between the ends of the capillary, the electric field exerts a force on the solution in the capillary as a whole. In an electric field, cations are attracted to the cathode and anions are attracted to the anode. Excess cations in the diffuse part of the double layer impart net momentum toward the cathode (Figure 3b). This pumping action, called *electroosmosis* (or *electroendosmosis*), is driven by cations within ~ 10 nm of the walls and creates uniform plug-like *electroosmotic flow* (EOF) of the entire solution toward the cathode. This process is in sharp contrast with *hydrodynamic flow*, which is driven by a pressure difference. In hydrodynamic flow, the velocity profile through a cross section of the fluid is parabolic: it is fastest at the centre and slows to zero at the walls (Figure 4).

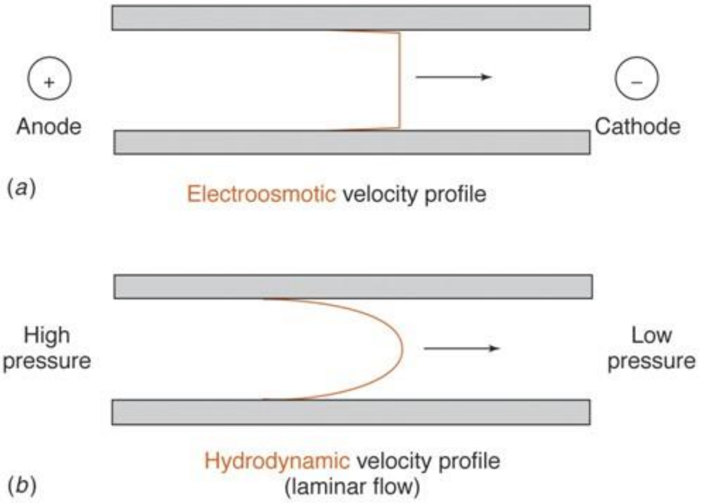


Figure 4. (a) Electroosmosis gives uniform flow over more than 99.9% of the cross section of the capillary. The speed decreases immediately adjacent to the capillary wall, (b) parabolic velocity profile of hydrodynamic flow (also called laminar flow), with the highest velocity at the centre of the tube and zero velocity at the walls [7].

The constant of proportionality between electroosmotic velocity, v_{eo}, and applied field is called *electroosmotic mobility*, μ_{eo}.

Electroosmotic mobility: $v_{eo} = \mu_{eo}E$

Electroosmotic mobility is proportional to the surface charge density on the silica and inversely proportional to the square root of ionic strength. Electroosmosis decreases at low pH ($Si\text{-}O^- \rightarrow Si\text{-}OH$ decreases surface charge density) and high ionic strength. Uniform electroosmotic flow contributes to the high resolution of capillary electrophoresis. Any effect that decreases uniformity creates band broadening and decreases resolution. The flow of ions in the capillary generates heat (called *Joule heating*) , which is not a serious problem in a capillary tube with a diameter of 50 µm, but the temperature gradient would be prohibitive if the diameter were in the order of millimetres. Some instruments cool the capillary to reduce the electrical conductivity of solution inside the capillary and prevent runaway Joule heating [7].

2.3 ANALYTICAL CONDITIONS

As previously mentioned, the achievement of high sensitivity is of the highest importance in forensic analysis. The analytical conditions implemented to fulfil the required method sensitivity mostly differ according to the detection method coupled to the CE electropherograph. For example, analytical sensitivity will depend greatly on analyte concentration in UV detection and on the mass of analyte which can be injected (into the CE system) in MS detection.

This subject will be dealt with in the present paragraph, where the attention will be focused on two general phases of the CE analysis: *i.* the sample pre-treatment required to concentrate low concentration/degraded samples; *ii.* sample injection modes in CE. Nevertheless, both sample pre-treatment and injection strategies apply to CE independently on the detection mode chosen.

Sample preparation is a crucial part of the analytical process, particularly in CE in which the co-existence of ions with highly different mobility and concentration may lead to disturbances of the separation process, e.g. peak defocusing. The purpose of any sample preparation is the clean-up of the sample in order to extract and/or pre-concentrate the substance/s of interest with the final aim of submitting to the separation process only a selected fraction of the sample containing all the molecules of interest and excluding all possible interferents. Sometimes, it may also be necessary to transform the analytes into different chemical forms, that can facilitate their separation and/or detection [11]. To these general considerations, when the focus of discussion is on CE, another aspect is worth mentioning. CE deals with minute amounts of samples and therefore it is particularly suitable for the application of the so-called *micro-extraction* techniques, which have been developed in different forms (single drops, fibres, hollow fibres, packed sectors of the capillary, etc.) [12-16]. Unfortunately, very few of these methods have so far been used in practice in forensics.

Moreover, it has to be taken into account that the choice of any sample pre-treatment will depend on the sample nature, the analyte characteristics and the subsequent analytical separation technique that is going to be employed, sometimes requiring a case-by-case development. Therefore, no universal sample preparation is available. Ideally, sample preparation steps should be kept to a minimum not only because this would reduce the analytical time and complexity, but also because the greater the number of steps, the higher the probability of introducing errors and loss of analytes [17].

Differently from liquid and gas chromatography, in CE, sample preparation has also the task of removing salts from the biological samples, which, as previously mentioned, cause peak defocusing. In addition, inorganic salts hinder the application of sample injection techniques based on electrostatic force (i.e. electrokinetic injection, filed amplified sample stacking, etc. that will be shortly described).

As previously mentioned, forensic samples are often found in low amounts and/or in the form of highly complex matrices, sometimes degraded (e.g. biological specimens in the crime scene), making crucial also the sample collection. For the aforementioned reasons, the choice of sample preparation in forensic analysis may result even more difficult than in clinical analysis, where samples are, in general, highly standardised and well preserved. Since forensic analysis deals also with non-biological materials (clandestine preparations of drugs, explosives, pharmaceuticals, etc.), forms of sample pre-treatment (from simple dilution to SPE, etc.) may be required, although less usually, in these cases and carefully selected.

With regards to sample injection, there are mainly two different classes of methods to introduce the sample into the first small part of the capillary: pressure- or electrostatic- driven modes.

In pressure-driven sample introduction, a pressure difference is applied between the inlet of the capillary, in contact with the sample solution, and the outlet. The pressure difference may be accomplished by gas pressure on the sample vial, referred to as *hydrodynamic injection*. However, the pressure difference may also be achieved by vacuum suction of the capillary end (known as *vacuum injection*) or by a height difference between then inlet and the outlet. When injection is led by pressure, the sample flows into the capillary with a parabolic flow profile.

$$\textit{Hydrodynamic injection:}\ \text{Volume} = \Delta P \pi d^4 t / 128 \eta L_t$$

where ΔP = pressure difference between the ends of the capillary

d = inner diameter of the capillary

t = injection time

η = sample viscosity,

L_t = total length of the capillary

On the other hand, sample injection techniques based on electrostatic force, when properly applied, may increase the analytical sensitivity by factors up to 1,000 (and more). Achieving this goal on-line in a narrow bore capillary is not an easy task and requires special attention. The simplest approach is just by applying electrokinetic injection, which by electrostatic force, draws the analytes with a suitable charge from the sample solution into the capillary inlet. Wey et al. reported the use of electrokinetic injection for CZE-MS of opioids in urine and urinary extracts [18]. Electrokinetic injection was found to provide higher sensitivity than hydrodynamic injection by a factor of over 1,000.

In electrokinetic injection, a voltage (usually lower than the one applied during the actual separation) is applied for a short time while the inlet of the capillary is submerged in the sample vial. The sample enters the capillary by a combined effect of osmosis and electrophoresis. This implies that the amount of analyte injected depends on its mobility, hence an analyte with a mobility in the same direction as the osmotic flow occupies a longer zone than one migrating in the opposite direction.

A sophisticated improvement is represented by head column field-amplified sample stacking (FASS), which, based on an electrolyte binary system, proved to be a robust approach to enhance sensitivity. After replenishing the capillary with the running buffer, a short low-conductivity zone (water plug) is introduced at the inlet side prior to electrokinetic injection from a sample solution of low conductivity. During electrokinetic injection, charged solutes migrate rapidly through the water zone, because of a very high local electric field. Upon reaching the interface with the running buffer, their migration velocity is drastically decreased because of the drop in the electric field due to a local higher conductivity. As a result, charged solutes are effectively concentrated at this interface, prior to their electrophoretic separation [18].

Another approach that can be adopted to increase sensitivity in CE, which also stems from electrokinetic injection, is the so-called *large volume sample injection*. In this scheme, an important part of the capillary is filled with the sample dissolved in a low conductivity buffer. After voltage application, because of a higher electric field in the sample plug than in the separation capillary, electrolytes with the suitable charge run faster until the interface with the separation buffer, where, because of a lower electric field, they stack in a small band, and the separation starts.

A further electrokinetic method to increase dramatically the sample load is the so-called "sweeping" which also applies to non-ionic species. The application of the on-line sample concentration sweeping technique to MEKC-MS was investigated by Isoo et al. to enhance the concentration sensitivity of environmental pollutants, such as several organic amines and alkyl phthalates [19]. In this study, both neutral (in the presence of EOF) and acidic (with suppressed EOF) conditions were applied and their sensitivities compared. Under the neutral conditions, a 40- to 100- fold gain in the concentration sensitivity was attained in the sweeping-MEKC-MS system compared to the conventional MEKC-MS system, whereas under the acidic conditions, a 100- to 600- fold sensitivity enhancement was achieved.

2.4 SEPARATION MODES

2.4.1 CAPILLARY ZONE ELECTROPHORESIS (CZE) AND NON-AQUEOUS CAPILLARY ELECTROPHORESIS (NACE)

CZE, usually performed in aqueous phase, is the original and still most common CE separation technique. Separation is based on differences in electrophoretic mobility. If the capillary wall is negative, electroosmotic flow is toward the cathode and the order of elution is cations before neutrals before anions. If the capillary wall charge is reversed by coating it with a cationic surfactant and the instrument polarity is reversed, then the order of elution is anions before neutrals before cations. Neither scheme separates neutral molecules from one another. CZE is the most common separation mode particularly when coupled with mass spectrometry.

NACE is, in principle, even more compatible with mass spectrometry, because of the utilisation of volatile solvents, being hence particularly suitable for analysis of compounds poorly soluble in water [20]. For this reason, in some instances, it can be considered a valid alternative to CZE in aqueous buffers.

For example, Jussila et al. used NACE-ESI-ion trap MS for the detection of methadone, pentazocine, levorphanol, dihydrocodeine and morphine [21]. The study introduced a modified type of interface, where the ESI spray capillary is extremely thin so that its inlet end is inserted into the CE capillary. Also methamphetamine, alprenolol, and levorphanol were detected by NACE by means of a sheathless nano-ESI interface based on liquid junction. Again, the interface was modified with a thin spraying needle in order to allow for the coupling of a wide-bore CE capillary with ion trap MS [22].

Capillary wall coating is a usual procedure in CZE, particularly when dealing with macromolecules, in order to avoid analyte adsorption onto the inner surface of the capillary, having a detrimental effect on the separation. Despite the almost universal use of physically adsorbed coatings in CZE with optical detection (since they can easily be applied, removed or replaced), chemically bonded coatings are preferred in CE-MS. This preference allows to avoid leaking of the coating material into the ion source, which may produce background noise, suppression of analyte signal, and/or contamination of the ion source and MS optics [23].

Notwithstanding this problem, Vanhoenacker et al. analysed six benzodiazepines in urine samples with CZE-ESI-ion trap MS and CZE-MS/MS by using a buffer system that dynamically coated the inner wall of fused silica capillaries (CEofix^{TM}, Analis, Suarlée, Belgium) [24]. According to the procedure, a buffer containing a polycation ("initiator") is flushed through the capillary. The polycations adsorb strongly to the capillary wall due to charge interactions. The capillary is then flushed with the running buffer containing a polyanionic additive (called "accelerator"). These polyanions interact with the lipophilic part of polycations forming a double layer. The polyanion layer contains sulphate groups and is rather insensitive to pH variations. Therefore, a stable and pH independent EOF is assured, when an electric field is applied across the capillary. After the analysis, the coating is stripped from the wall by a short rinse with NaOH followed by water. The use of such dynamic coating produced a high, constant and pH-independent EOF, without appreciable negative side effects on the MS side.

A similar dynamic coating was employed by Kohler et al. for the screening procedure of a two-step forensic toxicology analysis [25]. CZE interfaced with time-of-flight MS (CZE-ESI-TOF MS) was used for the screening step, requiring only urine dilution and online sample pre-concentration with pH-mediated stacking. Subsequently, quantitation of positive samples was performed by CZE-ESI-QqQ MS. The commercial dynamic coating CEofix^{TM} was employed only for the screening experiments, proving compatible with MS detection. Instead, an uncoated bare fused capillary was used for quantitative experiments.

2.4.2 CAPILLARY ISOTACHOPHORESIS

Capillary isotachophoresis (CITP) utilises a discontinuous buffer system comprised of a leading electrolyte (LE) and a terminating electrolyte (TE). The LE and TE have electrophoretic mobilities that are higher and lower, respectively, than the analytes of interest in the sample. The sample is located between the two electrolyte solutions and the analytes stack according to their electrophoretic mobilities (from the highest to the lowest) after the LE zone. Once the zones reach their steady state, the entire system moves at constant velocity, hence the name ‘‘iso’’ for same and ‘‘tacho’’ for speed [26].

ITP-TOF MS and ITP-CZE-TOF MS were used for the analysis of angiotensin peptides even at trace concentrations (0.3 μmol/L). The sample loading capacity in both ITP and ITP-CE was impressively high (up to 10 μL), if compared with the typical nanolitres-sized injection volumes of CE. It was concluded that ITP-TOF MS alone was adequate for the separation and detection of high concentration samples. The outcome was different at lower analyte concentrations where formation of mixed zones occurred, producing ion suppression and miss mass assignment. This problem was overcome by adding a CZE capillary between the ITP and TOF MS. In such an arrangement, samples were pre-concentrated in the high load ITP capillary and then transferred into the CZE capillary, where they were separated into non-overlapping peaks prior to their detection by TOF MS. The advantage of this integrated arrangement is that there is no need to discard portions of the sample in order to avoid overloading of the CE capillary. The authors concluded that this approach can be valuable for the analysis of complex samples with wide ranges of component concentrations [26].

CITP, indeed, is most often used as a sample focusing technique in several CE separation modes. Just to give an example, on-line CITP sample focusing allowed for loadability enhancement in capillary electro-chromatography fitted with UV or mass spectrometry (single quadrupole) detection. Its application to the analysis of small drugs such as salbutamol, fenoterol, neostigmine, and scopolamine in plasma and urine samples, provided sensitivities in the low nmol/L range [27].

2.4.3 MICELLAR ELECTROKINETIC CHROMATOGRAPHY

In micellar electrokinetic chromatography (MEKC), two distinct phases, an aqueous and a micellar phase, are used, the latter acting as a pseudo stationary phase. This phase is established by adding surfactants, such as SDS (sodium dodecyl sulphate), to an aqueous buffer above their critical micellar concentration (CMC) [28].

Being based on both electrophoretic and “chromatographic” principles, MEKC is suitable for the separation of charged compounds as well as neutrals, and therefore it allows for the analysis of a wide range of compounds of different nature, as it often occurs in forensic analysis. Clearly, the coupling of MEKC with MS seems extremely attractive and advantageous, as it would combine a highly versatile separation technique with mass-selective and structure-elucidative detection. However, the presence of high concentrations of surfactants in the separation electrolyte *per se* may hinder the MEKC-MS coupling, because of possible poisoning of the ion source [29].

A strategy to overcome the problem is to use a “partial filling” technique, in which the micellar phase is present only in the first part of the capillary, thus producing analyte separation without introduction of surfactant into the ion source. Stubberud et al. employed this approach for the analysis of ibuprofen, codeine, and their relative degradation products and impurities [30]. Specifically, by employing partial filling (PF)-MEKC-MS (single quadrupole), the separation and identification of all the compounds of interest was achieved, preventing the ion source contamination. First, the analytes migrated through the micelle zone where they were separated; subsequently, the migration continued through the electrophoretic background electrolyte (BGE) without micelles, until the analytes reached the ion source. Then the potential was turned off, thus hindering the contamination of the ion source by the micelles.

2.4.4 CAPILLARY ELECTROCHROMATOGRAPHY

Capillary electro-chromatography (CEC) stems from the combination of two separations techniques, namely liquid chromatography (LC) and CZE. In fact, a capillary column containing an LC stationary phase is eluted by a mobile phase, which rather than by pressure, is pushed forward by the electric field, producing at the silica surface an effective EOF. The mixed mode separation, the higher sample capacity, and the greater “peak capacity” of CEC offer neat advantages *versus* CE. However the CEC column reproducibility is still questionable [31]. Mixtures of benzodiazepines, corticosteroids and thiazide diuretic drugs were analysed by Taylor et al. using UV absorbance and ESI-MS detection [32]. Separations were performed on fused-silica capillary columns packed with CEC Hypersil ODS (Thermo Fisher Scientific, Waltham, MA, USA) or Apex ODS particles (Jones Chromatography, Hengoed, UK).

2.4.5 CAPILLARY GEL ELECTROPHORESIS

Capillary gel electrophoresis (CGE) is a variant of gel electrophoresis, which has been a primary tool in biochemistry for four decades. Slabs of polymer gel used to separate macromolecules according to size have customarily been chemical gels, in which chains are cross-linked by chemical bonds. Chemical gels cannot be flushed from a capillary, so physical gels in which polymers are simply entangled are used. Physical gels can be flushed and reloaded to generate a fresh capillary for each separation. Macromolecules are separated in a gel by sieving, in which smaller molecules migrate faster than large molecules through the entangled polymer network. CGE is the method of choice to separate polymers formed by monomers with the same charge. Large biomolecules such as DNA, RNA, and proteins are typically analysed by using CGE [31].

2.4.6 CE ON A MICROCHIP

An advantage of CE is that the core of instrumentation can be miniaturised in a microchip format. In this configuration, the consumption of sample and reagents, the analysis time and the costs may be reduced to the minimum. In addition, analytical speed and instrument portability are also increased [33,34]. The chips can be made of various material among which glass, silicon [34] and polymer, such as polydimethylsiloxane [35]. The epoxy-based negative photoresist SU-8 has shown to be a very suitable material for microchip production. Sensitive and efficient drug screening of urine samples was reported by using the SU-8 CZE-ESI-QqQ MS microchips (Figure 5). The microchip method including rapid CZE separation followed by MS detection was applied to two parent drugs, paracetamol and tramadol, as well as to their metabolites (glutathione-, glucuronide-, and mercapturate-paracetamol; O-desmethyl-, and N-desmethyl- tramadol) [36].

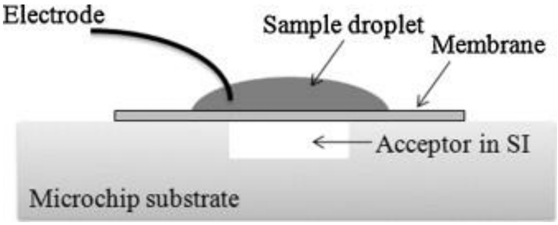


Figure 5. Schematic illustration of the on-chip droplet-membrane-droplet LPME system on top of an SU-8 CE-ESI/MS microchip. SI = sample inlet [36].

3. CHIRAL SEPARATIONS IN CE

Chirality is a promising topic in analytical sciences since the biological activity of a pair of enantiomers can severely differ. Importance of chirality is reflected in many fields especially in pharmaceutical, food, environmental, clinical, and toxicological areas as shown in recent literature [37]. In most of these applications, a high sensitivity is required in order to detect or even quantify trace or ultra-trace components. Consequently, sensitive analytical methodologies are needed to solve problems in a wide variety of areas. Among the analytical techniques used in chiral analysis, CE emerges as a very useful tool to carry out stereoselective separations thanks to not only its high specificity, flexibility and all the advantages derived from the small dimensions of the separation capillary but also thanks to the availability of many types of chiral selectors. Thus, numerous methods in CE have been developed for the analysis of chiral compounds in chemical, biochemical, pharmaceutical, environmental, and forensic sciences.

Moreover, it is worth highlighting that, in CE, unlike in chromatographic techniques, the chiral selector is generally within the separation medium instead of being attached to the separation column resembling a pseudo stationary phase. This may possess its own mobility allowing different separation scenarios, which makes the method optimisation more versatile and contributes to the high flexibility of CE.

Regardless of its numerous advantages, when using UV-Vis detection, CE lacks of detection sensitivity due to its short optical path length derived from the narrow separation capillary. This issue can be overcome by means of different approaches, either by sample treatment procedures or by in-capillary pre-concentration techniques or even by employing detection systems more sensitive than UV-Vis, such as LIF or MS [6].

Despite a vast availability of different types of chiral selectors, most reported applications in CE deal with cyclodextrins (CDs). CDs are cyclic oligosaccharides prepared form starch by enzymatic degradation. They have a shape of a truncated cone with a relatively hydrophobic open cavity, and a hydrophilic outer layer due to the presence of hydroxyl groups. Although CDs containing between 6 to 12 glucopyranose units have been isolated, only those containing six (α-CD), seven (β-CD) or eight (γ-CD) residues are currently used in analytical chemistry (Figure 6). The physical properties of the three native CDs differ from each other by some features such as width of the cavity, solubility, molecular mass, etc. but they possess the same depth [38].

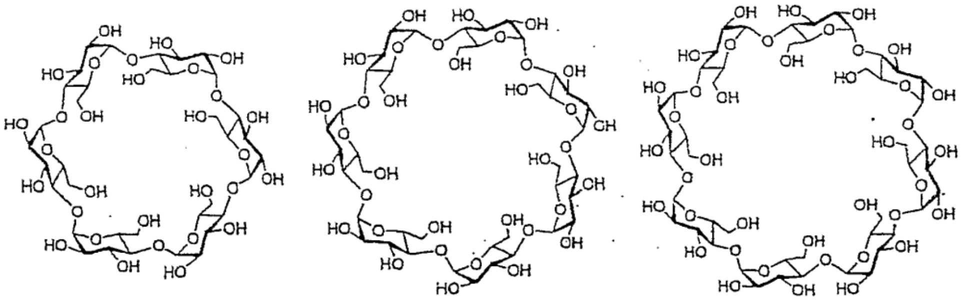


Figure 6. Structure of α-CD, β-CD and γ-CD.

The size of the hydrophobic cavity is such that, in general, the α-CD can accommodate a single phenyl ring, while β-CD and γ-CD can accommodate substituted single- and multiple- ring systems.

In general, analytes fit the cavity either with the whole molecule or with their hydrophobic part forming inclusion-complexes stabilised by secondary bonds between the rim of the chiral selector and substituent groups of the analyte asymmetric centre. The complex formed during the electrophoretic process, in equilibrium with the free analyte, possesses a different mass responsible for the change of the effective mobility.

Since the inclusion-complexation mechanism is based on the possibility that a certain analyte fits into the cavity of the CDs, the shape and the size of both host and guest molecule are of relevant importance in such mechanism.

Besides the already mentioned native cyclodextrins, a wide range of modified cyclodextrins is commercially available. The hydroxyl groups present on the rim of the CDs can be easily modified by chemical reactions in order to obtain CD derivatives with a different degree of substitution. Such modifications of the native cyclodextrins observably lead to a different stereoselectivity, but also to an improved solubility, possibility for different secondary bonds, different hydrophobicity of the cavity, etc. Charged or chargeable groups can also substitute hydroxyl groups on the cyclodextrins rim. The introduction of chargeable groups will result in an increased solubility and will allow the separation of uncharged species [39]. Furthermore, the use of chiral selectors carrying a charge opposite to that of the analytes can greatly improve the mobility difference between the two optical antipodes.

In particular, two classes of negatively charged CDs were employed in our studies. Namely sulphated β CDs (S-β-CD) and highly sulphated γ CDs (HS-γ-CDs), they are derived from native β and γ, respectively, by sulphation. The combination of these anionic additives with the high efficiency of CE proved in literature a versatile and generally applicable separation scheme [5,40,41].

To cite a few examples, Merola et al. used β-CD and HS-γ-CD for achieving the chiral separation, by means of CZE-MS, of 12 cathinone analogues, belonging to the benzoylethanamines class and sold illegally as “bath salts” [42]. These compounds differ from one another by the presence of substituents that may include halogen, dioxol, and alkyl groups; hence, a highly efficient and selective method for the discrimination of such closely related compounds is needed. The procedure used β-CD for CZE-UV analysis and HS-γ-CD for CZE-MS. The method provided chiral resolution and identification of individual analytes present in the mixture by determination of the exact mass by TOF MS. The method was tested using a small set of commercial “bath salts” and finally applied to seized drug sample.

HS-γ-CDs were also the chiral selectors of choice for the simultaneous chiral separation of nine amphetamine-type stimulants by Reverse Polarity (RP) CZE with electrospray positive ionisation QTOF MS. This method was also applied to the analysis of trace precursors in illicit methamphetamine seizures [43].

Sulphated β-CDs proved most effective for the chiral separation of anaesthetic drugs including bupivacaine, mepivacaine, prilocaine and ketamine, according to

Cherkaoui et al. who reported the on-line coupling of CZE with ESI–MS, using the counter current PF (partial filling) technique [44]. The optimised method was successfully applied to the enantioseparation of mepivacaine in injectable solutions.

Finally, the separation of four basic drugs (methadone, fluoxetine, venlafaxine, and tramadol) in standard solutions was obtained using low concentration of HS-γ-CDs in normal polarity using PF-CZE-ESI-MS [45]. The same research group used the same chiral selector for the analysis of plasma samples containing seven amphetamines [46].

4. MASS SPECTROMETRY

It is supposed that mass spectrometry originated in 1886, when Golstein, a German physicist, discovered that positive ions generated from an electric field to which a low pressure was applied. In 1898, Wien showed that a beam of such ions could be deflected by an electric field and a magnetic field. Subsequently, between 1912 and 1919, Thompson and Aston improved the device dedicated to the study of such ions, which resulted in the first "mass spectrograph" in 1919. The instrument could separate ions differing in mass by as little as 1% and was extremely valuable for physicists for the study of atomic structures. Aston immediately found that neon consists of two isotopes (^{20}Ne and ^{22}Ne) and went on to discover 212 of the 281 naturally occurring isotopes. He received the Nobel Prize for chemistry in 1922 [7].

Mass spectrometry has long been used to measure isotopes and decipher organic structures. It can elucidate biomolecule sequences, such as the amino acid sequence in a protein, the sequence of nucleic acids in DNA, the structure of a complex carbohydrate, and the types of lipids in a single organism. Mass spectrometry can measure masses of individual cells and classify cells [47]. Moreover, it is the most powerful detector for chromatography, offering both qualitative and quantitative information, providing high sensitivity, and distinguishing different substances with the same retention time.

"The basic principle of mass spectrometry is to generate ions from either inorganic and organic compounds by any suitable method, to separate ions by their mass-to-charge ratio (*m/z)* and to detect them qualitatively and quantitatively by their respective *m/z* and abundance. The analyte may be ionised thermally, by electric fields, or by impacting energetic electrons, ions or photons. The so-formed ions can be single ionised atoms, clusters, molecules or their fragments or associates. Ion separation is effected by static or dynamic electric or magnetic field." Although this definition dates back to 1968, it is still valid with some additions to be made. Firstly, ionisation of a sample can be made not only by electrons, but also by (atomic) ions or photons, energetic neutral atoms, electronically excited atoms, massive cluster ions, and even by electrostatically charged micro droplets. Secondly, ion separation by *m/z* can also be effected in field-free regions for those ions which possess a well-defined kinetic energy at the entrance of the flight path, as greatly demonstrated by the time-of-flight (TOF) analyser [47].

Mass spectrometry is a technique for studying the masses of atoms or molecules or fragments of molecules. In order to obtain a mass spectrum, gaseous species desorbed from condensed phases are ionised; the ions are accelerated by an electric field and then separated according to their *m/z*. If all charges are +1, then *m/z* is numerically equal to the mass. If an ion has a charge of +2, for example, then *m/z* is 1/2 of the mass [7]. As a result, a mass spectrum displays detector response versus *m/z*, where the area of each peak is proportional to the abundance of each isotope.

A magnetic sector mass spectrometer, possibly the most historically known type of mass spectrometer, uses a magnetic field to allow ions of a selected *m/z* to pass from the ion source to the detector. Gaseous molecules entering the ion source are converted into ions (usually positive ions), accelerated by an electric field, and expelled into the analyser tube, where they encounter a magnetic field perpendicular to their direction of travel. The tube is maintained under high vacuum so that ions are not deflected by collision with background gas molecules. The magnet deflects ions toward the detector at the far end of the tube [48]. Heavy ions are not deflected enough and light ones are deflected too much to reach the detector. The spectrum of masses is obtained by varying the magnetic field strength. At the electron multiplier detector, each arriving ion starts a cascade of electrons, just as a photon starts a cascade of electrons in a photomultiplier tube (Figure 7). A series of dynodes multiplies the number of electrons by ~ 10^5 before they reach the anode where current is measured. The mass spectrum shows detector current as a function of *m/z* selected by the magnetic field.

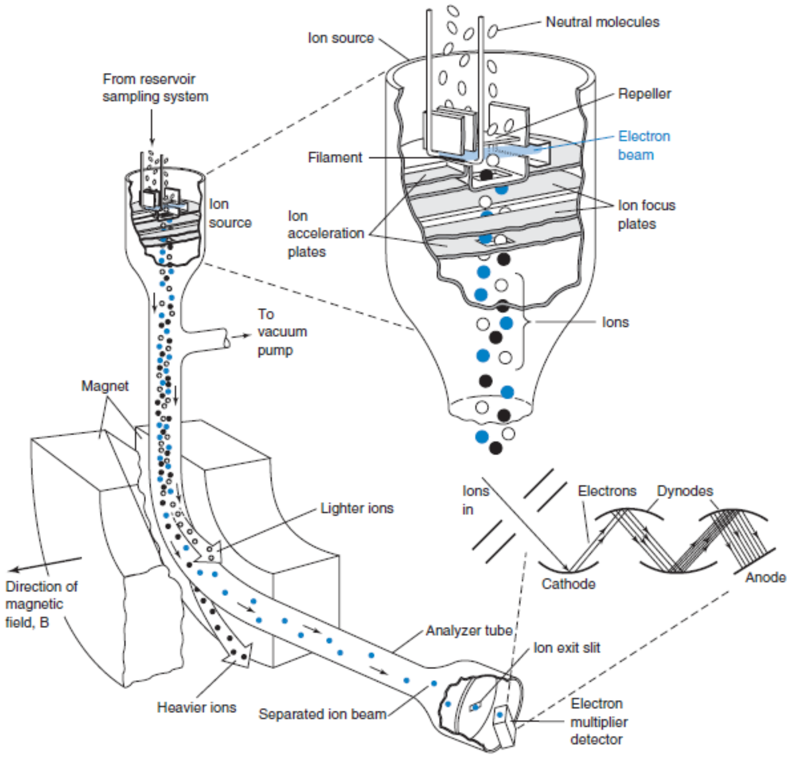


Figure 7. Schematic example of magnetic sector mass spectrometer [7].

Mass spectrometers work equally well for negative and positive ions by reversing voltages where the ions are formed and detected. To detect negative ions, a conversion dynode with a positive potential is placed before the conventional detector. When bombarded by negative ions, this dynode liberates positive ions that are accelerated into the electron multiplier, which amplifies the signal.

4.1 IONISATION SOURCES

There is a wide variety of ionisation techniques available up to date with their relative key applications. Approximately, they can be classified by their relative hardness or softness and by the molecular mass of suitable analytes.

In the system described in the previous paragraph, molecules entering the ion source are converted into ions by electron ionisation. Electrons emitted from a hot filament (like the one in a light bulb) are accelerated through 70 V before interacting with incoming molecules. Some molecules (M) absorb as much as 12–15 electron volts, which is enough for ionisation:

$$M + e^- \rightarrow M^{+\cdot} + e^- + e^-$$

Almost all stable molecules have an even number of electrons. When one electron is lost, the resulting cation with one unpaired electron is designated $M^{+\cdot}$, the molecular ion. After ionisation, $M^{+\cdot}$ usually has enough residual internal energy to break into fragments. A small positive potential on the repeller plate of the ion source pushes ions toward the analyser tube, and a small potential on the ion focus plates creates a focused beam. High voltage (~ 1,000–10,000 V) between the ion acceleration plates imparts a high velocity to ions as they are expelled from the bottom of the ion gun.

The electron kinetic energy of 70 eV is much greater than the ionisation energy of molecules. For example, in the case of formaldehyde, the most easily lost electron comes from a non-bonding (“lone pair”) orbital centred on oxygen, with an ionisation energy of 11.0 eV. To remove a *pi* bonding electron from neutral formaldehyde requires 14.1 eV, and to remove the highest-energy *sigma* bonding electron from the neutral molecule requires 15.9 eV. Interaction with a 70-eV electron will most likely remove the electron with lowest ionisation energy. The resulting molecular ion, $M^{+\cdot}$, can have so much extra energy that it breaks into fragments. There might be so little that its peak is small or absent in the mass spectrum. If you lower the kinetic energy of electrons in the ionisation source, to 20 eV for example, there will be a lower yield of ions and less fragmentation. You would likely observe a greater abundance of molecular ions.

The most intense peak in a mass spectrum is called the *base peak*. Intensities of other peaks are expressed as a percentage of the base peak intensity.

As previously mentioned, ionisation strategies are generally classified into two different groups: *hard* and *soft* ionisation techniques. The *hard ionisation* generates ions with high energy content and usually with an odd number of electrons (OE = Odd Electron ion, i.e. radical ion). Because of the high energy involved, generally extensive fragmentation likewise takes place. *Soft ionisation* gives rise to ions that are considerably more similar to the ones generated in aqueous solutions. In addition, a single ion is typically generated.

The first group includes:

- EI (electron ionisation)
- ICP (inductively coupled plasma)

Soft ionisation techniques include:

- CI (chemical ionisation)
- APCI (atmospheric pressure chemical ionisation)
- ESI (electrospray ionisation)
- LDI (laser desorption ionisation)

The most common methods will briefly be discussed in the following paragraphs.

4.1.1 ELECTRON IONISATION (EI)

Electron ionisation is historically the first developed ionisation technique and, possibly, the most commonly used. It is classified among the *hard ionisation techniques* because of the high energy involved. For the same reason, molecules impacted by such strategy not only ionise but, generally, also undergo fragmentation. In the ionisation chamber ($10^{-6} – 10^{-7}$ mm Hg), the molecules of the analyte of interest, in its gaseous phase, interact with an electron beam generated by an incandescent filament (Rhenium or Tungsten) and accelerated through a voltage controlled by the operator. The energy of the beam is commonly set at 70 eV. The interaction between electrons and the molecules of the analyte may create two opposite situations: *i*. the molecule loses an electron, giving rise to a positive radical ion; *ii*. the electron is gained by the molecule originating a radical anion. Due to energetic reasons, the fist scenario is most likely to occur. In fact, the acquisition in positive ion mode is the most widespread when EI is involved.

In general, as previously mentioned, the mechanisms of ionisation may be summarised as follows: the molecule, in its gaseous state impacts with an electron and gives rise to a radical cation and two electrons:

$$M + e^- \rightarrow M^{+\cdot} + e^- + e^-$$

The kinetic energy is transferred to the system generating an electron transition and reaching orbitals of levels high enough to allow the electron to be lost. The impacted molecule ($M^{+\cdot}$) is both a radical, due to the unpaired electron, and a cation because of a non-balanced proton. The $M^{+\cdot}$ species is called *molecular ion* and is highly unstable, therefore, because of the excess of energy undergoes further fragmentation. The fragments obtained show *m/z* ratios progressively lower since they may also be unstable and experience fragmentation into a series of molecules and/or neutral radicals, which are not detected, and a series of daughter ions that are separated and detected.

Such extensive fragmentation, however, appears to be highly reproducible, which may surely be included in the list of advantages of EI, together with its spectra repeatability, high efficiency hence high sensitivity.

On the other hand, only positive ions and radical cations are formed. Finally, because such ionisation source requires the analyte to be volatile, its applicability appears limited with regards to some biological matrices, despite most substances may be made more volatile through chemical derivatisation. Moreover, it is mostly adaptable to GC coupling rather than LC coupling although the latter still exists, especially in the form of nano-LC-EI coupling.

4.1.2 CHEMICAL IONISATION (CI)

Chemical ionisation, included in the soft ionisation techniques list, still produces fragmentation although less than electron ionisation. For chemical ionisation, the ionisation source is filled with a reagent gas such as methane, isobutane, or ammonia, at a pressure of ~1 mbar. Energetic electrons (100–200 eV) convert CH_4 into a variety of reactive products:

$$CH_4 + e^- \rightarrow CH_4^{+\cdot} + 2\ e^-$$

$$CH_4^{+\cdot} + CH_4 \rightarrow CH_5^+ + {}^{\cdot}CH_3$$

$$CH_4^{+\cdot} \rightarrow CH_3^+ + H^{\cdot}$$

$$CH_3^+ + CH_4 \rightarrow C_2H_5^+ + H_2$$

CH_5^+ is a potent proton donor that reacts with analytes to give the *protonated molecule,* MH^+, which is usually the most abundant ion in the chemical ionisation mass spectrum.

$$CH_5^+ + M \rightarrow CH_4 + MH^+$$

Ammonia or isobutane are used in place of CH_4 to reduce the fragmentation of MH^+. These reagents bind H^+ more strongly than CH_4 does and impart less energy to MH^+ when the proton is transferred to M. NO^+ is a mild, versatile ionisation reagent generated from NO by radioactive ^{210}Po. Negative chemical ionisation reagents include 0_2^- , F^-, and SF_6^-.

The possibility to use different gases make the fragmentation derived by CI more versatile, being adjustable although less reproducible.

4.1.3 ATMOSPHERIC-PRESSURE CHEMICAL IONISATION (APCI)

In atmospheric-pressure chemical ionisation, heat (at very high temperatures) and a coaxial flow of N_2 convert eluate into a fine mist, at the end of the capillary, from which solvent and analyte evaporate. Like chemical ionisation in the ion source of a mass spectrometer, atmospheric-pressure chemical ionisation creates new ions from gas-phase reactions between ions and molecules. The distinguishing feature of this technique is that a high voltage is applied to a metal needle in the path of the aerosol. An electric corona (a plasma containing charged particles) forms around the needle, injecting electrons into the aerosol and creating ions from nearby molecules. Atmospheric-pressure chemical ionisation handles a variety of analytes and accepts chromatography flow rates up to 2 mL/min. It is advantageous for its ability to easily ionise and detect non-polar or slightly polar species. Generally, in order to be observed, the analyte M must be capable of forming the protonated ion, MH^+. Atmospheric-pressure chemical ionisation tends to generate little fragmentation and to produce single-charge ions, which makes it unsuitable for macromolecules such as proteins.

4.1.4 ELECTROSPRAY IONISATION (ESI)

Pneumatically assisted electrospray, also called ion spray, is an ionisation source that originates a process of nebulisation by means of an inert gas (usually N_2) and is illustrated in Figure 8. Liquid from the chromatography column enters the nebuliser capillary, along with a coaxial flow of N_2 gas. For positive ion mass spectrometry, the nebuliser is held at 0 V and the spray chamber is held at -3,500 V. For negative ion mass spectrometry, all voltages would be reversed. The strong electric field at the nebuliser outlet, combined with the coaxial flow of N_2 gas, creates a fine aerosol of charged particles. Ions that vaporise from aerosol droplets were already in solution in the chromatography column. For example, protonated bases (BH^+) and ionised acids (A^-) can be observed. Other gas-phase ions arise from complexation between analyte, M (which could be neutral or charged), and stable ions from the solution. For example:

MH^+ (mass = M + 1)

$M(K^+)$ (mass = M + 39)

$M(NH^{4+})$ (mass = M + 45)

$M(CH^3CO^{2-})$ (mass = M + 59)

Positive ions from the aerosol are attracted toward the glass capillary leading into the mass spectrometer by an even more negative potential of – 4,500 V. Gas flowing from atmospheric pressure in the spray chamber transports ions through the capillary to its exit, where the pressure is reduced to ~ 3 mbar by a vacuum pump.

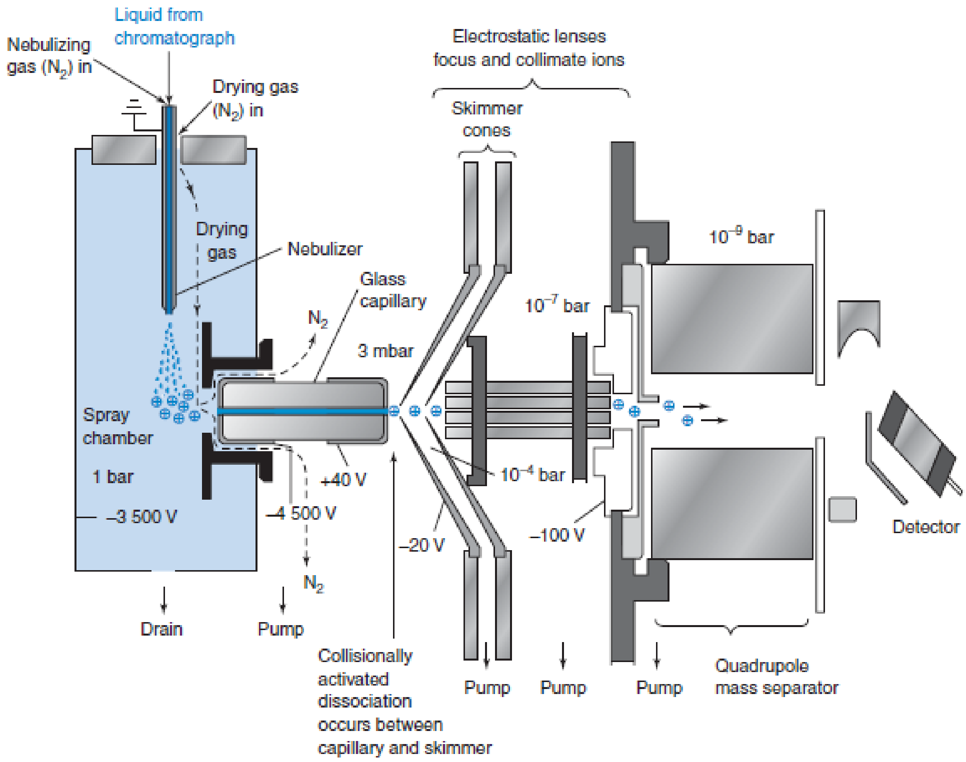


Figure 8. Pneumatically assisted electrospray interface [7].

Figure 9 provides more detail on ionisation. Voltage imposed between the nebuliser capillary and the inlet to the mass spectrometer creates excess charge in the liquid by redox reactions. Electrons from the oxidation flow through the external circuit and possibly neutralise gaseous positive ions at the inlet to the mass spectrometer. Charged liquid exiting the capillary forms a cone and then a fine filament to finally break into a spray of fine droplets. A droplet shrinks to ~ 1 µm by solvent evaporation until the repulsive force of the excess charge equals the cohesive force of surface tension. At that point, the droplet breaks up by ejecting tiny droplets with diameters of ~ 10 nm. They evaporate, leaving their cargo of ions in the gas phase.

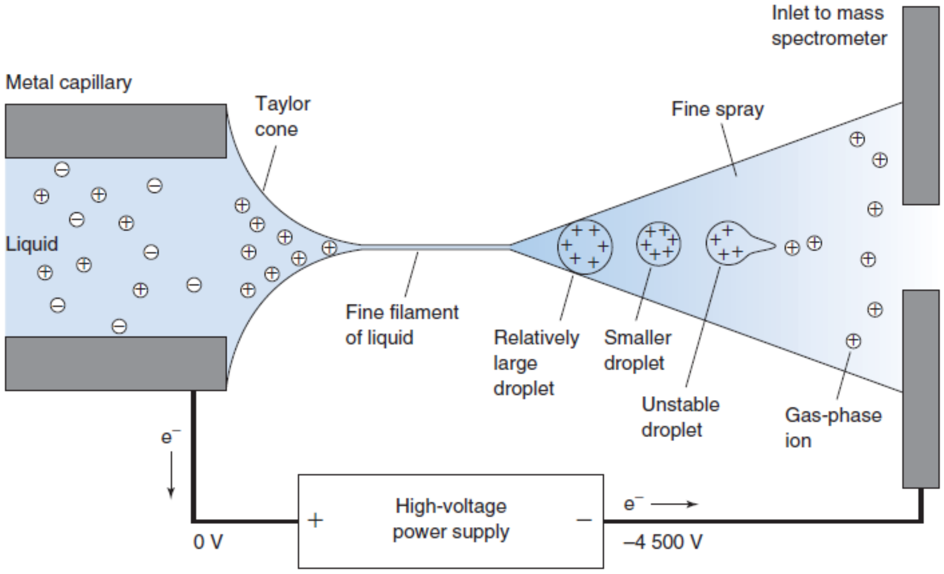
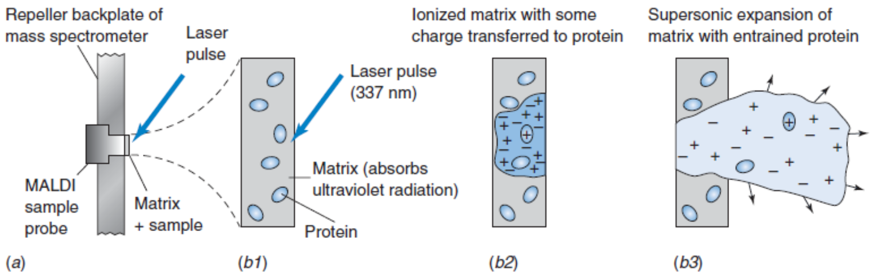


Figure 9. Gas-phase ion formation [7].

In electrospray, little fragmentation of analyte occurs and mass spectra are simple. Fragmentation can be intentionally increased by collisionally activated dissociation (also referred to as *collision-induced dissociation*) in the region between the glass capillary and the skimmer cone. Another advantage is that ESI enables the detection of large, non-volatile molecules (e.g. proteins). Finally, multiply charged molecules are formed mostly due to the competition between the forming ions and the solvent. However, when competition occurs between the forming ions and the salts in the ionisation source, the spectrum will negatively be influenced.

4.1.5 MALDI (MATRIX-ASSISTED LASER DESORPTION IONISATION)

Major methods for introducing proteins and other macromolecules into mass spectrometers are electrospray and matrix-assisted laser desorption/ionisation (MALDI). Most often, MALDI is used with a time-of-flight mass spectrometer, which can measure *m/z* up to 10^6. Typically, 1 µL of a solution of a 10 µm analyte is mixed with 1 µL of a 1–100 mM solution of a UV-absorbing compound such as 2,5-dihydroxybenzoic acid (the matrix) directly on a probe that fits into the source of the spectrometer. Evaporation of the liquid leaves an intimate mixture of fine crystals of matrix plus analyte. In principle, the desorption is triggered by the UV laser beam, which triggers the ionisation. In order to introduce ions into the gas phase for mass spectrometry, an IR or UV pulse from a laser is directed onto the sample. The matrix vaporises and expands into the gas phase, carrying analyte along with it (Figure 10). The high matrix/sample ratio inhibits association between analyte molecules and provides protonated or ionic species that transfer charge to analyte. Shortly after ions expand into the source, a voltage pulse applied to the backplate expels ions into the spectrometer. The resolving power is 10^3-10^4 and mass accuracy may reach 0.005–0.05%.



Sequence of events in matrix-assisted laser desorption/ionization. (*a*) Dried mixture of analyte and matrix on sample probe inserted into backplate of ion source. (*b1*) Enlarged view of laser pulse striking sample. (*b2*) Matrix is ionized and vaporized by laser and transfers some charge to analyte. (*b3*) Vapor expands in a supersonic plume.

Figure 10. Schematic illustration of ions formation in MALDI [7].

Like ESI, MALDI enables the detection of large, non-volatile molecules (e.g. proteins) nevertheless no formation of multiply charged molecules occurs.

4.2 MS ANALYSERS

The MS analyser is the portion of the mass spectrometer where the selection of ions, previously generated by the ionisation source, occurs. The physical property of ions measured by a mass analyser is their *m/z* ratio. Ions with different *m/z* values will reach the detector at different times, thus appearing in the mass spectrum in different regions. However, the separation of ions according to their *m/z* can be based on different principles. Nevertheless, all mass analysers use static or dynamic electric and magnetic fields, alone or combined. The magnetic sector is a static analyser, while time-of-flight (TOF), quadrupole (Q), ion trap (IT), ion cyclotron resonance (ICR) are dynamic analysers. All mass spectrometers combine ion formation, mass analysis and ion detection; however, each mass analyser has its own peculiar features valuable for a specific analysis type. Each mass analyser may show both benefits and limitations for a given application, yet its choice should be made upon the application, cost and performance. “There is no ideal mass analyser that is good for all applications” [49].

There are various classifications to distinguish different type of analysers but, in general, they can be divided into two broad classes based on many properties:

- Scanning analysers that transmit the ions of different masses successively along a time scale (magnetic sector or quadrupole instruments) *vs.* analysers that allow the simultaneous transmission of all ions (TOF, dispersive magnetic sector, IT)
- Ion beam (TOF, magnetic sector, Q) *vs.* ion trapping types (IT, FT-ICR, FT-Orbitrap)
- Continuous *vs.* pulsed analysis
- Low *vs.* high kinetic energies.

Prior to discuss the most common mass analysers in the following paragraphs, the main definitions used in mass spectrometric analyses will be briefly reported.

Atomic mass: weighted average of the masses of the isotopes of an element. For example, bromine consists of 50.69% ^{79}Br with a mass of 78.91834 Da and 49.31% ^{81}Br with a mass of 80.91629 Da. Therefore, its atomic mass is (0.5069) x (78.91834) + (0.4931) x (80.91629) = 79.904 Da. The unit of atomic mass is the dalton, Da, defined as 1/12 of the mass of ^{12}C. In MS, the symbol “u” for “unified atomic mass unit” and Da are synonymous.

Molecular mass (of a molecule or ion): sum of atomic masses listed in the periodic table. For example, for bromoethane C_2H_5Br, the molecular mass is (2 x 12.0107) + (5 x 1.00794) + (1 x 79.904) = 108.965.

Nominal mass (of a molecule or ion): is the *integer* mass of the species with the most abundant isotope of each of the constituent atoms. For carbon, hydrogen, and bromine, the most abundant isotopes are ^{12}C, ^{1}H, and ^{79}Br, respectively. Therefore the nominal mass of C_2H_5Br is (2 x 12) + (5 x 1) + (1 x 79) = 108.

Mass limit: value of *m/z* above or below which ions cannot be detected in a mass spectrometer.

Mass range: range of mass in which a mass spectrometer can detect ions or is operated to record a mass spectrum.

Dynamic range: ratio of the largest detected peak to the smallest detected peak in the mass spectrum. Typically higher on beam instruments than on trapping analysers.

Transmission: ratio of the number of ions reaching the detector and the number of ions entering the mass analyser.

Signal to noise (S/N): ratio of the magnitude of a specific peak (or highest magnitude peak), in the spectrum, to the noise level. The noise level must be specified for a useful comparison.

Mass resolution: by one definition, the mass resolution is $m/\Delta m$, where the denominator is the separation of the two peaks when the overlap at their base is 10% as high as the peak. By a second definition, the mass resolution is $m/m_{1/2}$, where $m_{1/2}$ is the width of the peak at half the maximum height (Full Width at Half Maximum definition, FWHM).

Mass resolving power: measure of the ability of a mass spectrometer to provide a specified value of mass resolution. The higher the resolving power of a mass spectrometer, the better it is able to separate two species with similar *m/z* values.

Mass accuracy: indicates the accuracy of the *m/z* provided by the mass analyser, it is the difference that is observed between the theoretical and the measured *m/z.*

4.2.1 QUADRUPOLE (Q)

Quadrupole analysers are made up of four round rods (or, ideally, hyperbolic rods), that must be perfectly parallel, whose centres form the corners of a square and whose opposing electrode pairs are connected (Figure 11).

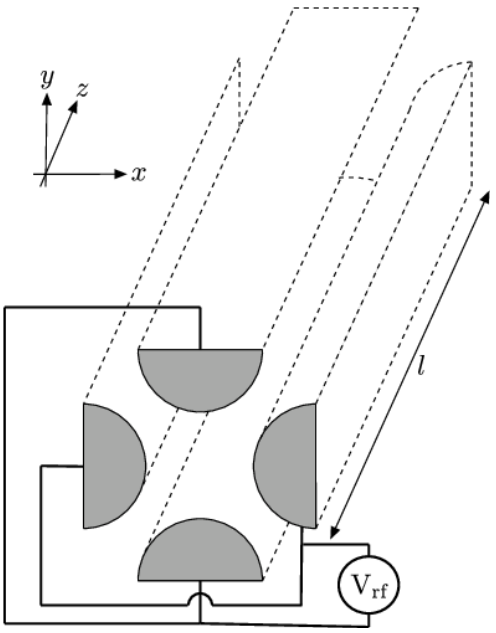


Figure 11. Schematic positioning of the rods of a quadrupole.

Both a constant voltage and a radiofrequency oscillating voltage are applied between the connected pairs of rods. Ions, thanks to the electric field will be deflected in complex trajectories, during their migration in the analyser, which may be stable or unstable. Stable oscillations allow only ions with one particular *m/z* ratio to leave the quadrupole and reach the detector; other ions (non-resonant ions) will collide with the rods of the quadrupole and will be lost before they reach the detector. However, the value of the applied voltage may be varied in a way that selected ions of different mass may reach the detector at different, specific times.

Because of their low cost, quadrupole analysers are the most common separators and, usually are operated at *unit resolution*, meaning that the resolution is sufficient to separate two peaks one unit mass apart.

4.2.2 ION TRAP (IT)

The ion-trap mass spectrometer is similar to the Q analyser but the quadrupolar filter in it is spherical, hence the generated ions will progressively be released towards the detector by varying the electric field. One of the most widely used mass separators, the IT is a compact device that is well suited as a chromatography detector. In the internal ionisation quadrupole ion trap, substances emerging from the chromatography column enter the cavity of the mass analyser through a heated transfer line. Molecules undergo electron ionisation in the cavity formed by the two end caps and a ring electrode, all of which are electrically isolated from one another. Alternatively, chemical ionisation is achieved by adding a reagent gas such as methane to the cavity.

A constant-frequency, radio-frequency voltage applied to the central ring electrode causes ions to circulate in stable trajectories around the cavity. Increasing the amplitude of the radio-frequency voltage expels ions of a particular *m/z* value by sending them into unstable trajectories that pass through the exit holes in the end caps. Ions expelled through the lower end cap are captured by the electron multiplier and detected with high sensitivity.

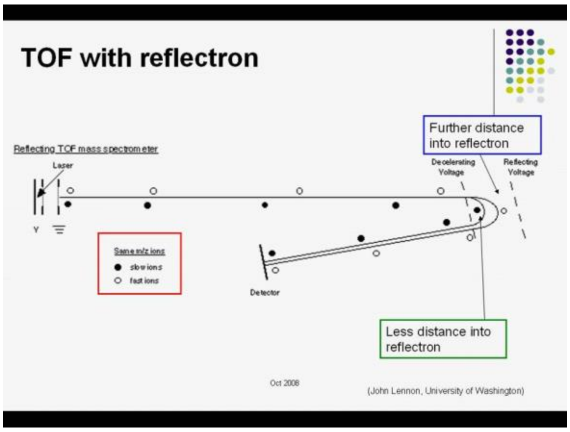
In other mass analysers, only a small fraction of ions reaches the detector. With an ion trap, half of the ions of all *m/z* values reach the detector, giving this spectrometer 10–100 times more sensitivity than the transmission quadrupole.

4.2.3 TIME-OF-FLIGHT (TOF)

The principle of the time-of-flight mass analyser is that, ideally, all ions have the same kinetic energy 1/2 mv^2 (where m is the mass of the ion and v is its velocity), but different masses. Ultimately, ions are separated in a field-free region after acceleration through a fixed accelerating potential applied in the ionisation chamber. The region where the separation occurs is a long, straight, evacuated tube with the source at one end and the detector at the other end. It appears clear that ions with different *m/z* will require different times to move along the given distance in the field-free region, with the lighter ones travelling faster than the heavier ones.

In this manner, TOF analysers allow to detect “all” ions that enter the flight tube with a full *m/z* range acquired, which makes analysis speed elevated.

Being the resolving power dependant on time and on the length of the tube in this system, the improvement of TOF analysers was achieved in time with the introduction of the *reflectron* system. The reflectron is a series of hollow rings held at increasingly positive potential, terminated by a grid whose potential is more positive than the accelerating potential on the back plate of the source (Figure 12). It is employed to overcome the kinetic energy distribution of ions with the same *m/z* value. Ions entering the reflectron are slowed down, stopped, and reflected. The more kinetic energy an ion had when it entered the reflectron, the farther it penetrates before it is turned around. Reflected ions reach a new space focus plane at the grid in front of the detector. All ions of the same mass reach this grid at the same time, regardless of their initial kinetic energies.


TOF with reflectron

Further distance into reflectron

Less distance into reflectron

Oct 2008

(John Lennon, University of Washington)

Figure 12. Representation of the reflectron in the TOF analyser.

5. APPLICATIONS

5.1 FORENSIC TOXICOLOGY

The term “forensic toxicology” covers those applications of the biomedical and analytical sciences aimed at the elucidation of the toxicological problems occurring in the criminal investigation, in the penal trials (e.g. poisoning), as well as in the civil litigation (e.g. child custody, divorce) and in different administrative fields (e.g. fitness to drive or to work) [33].

In forensic toxicology, according to a well established international agreement, a two-step approach is used in the analysis of biological (and complex non-biological) samples (Figure 13). The first step usually consists of a rapid screening, generally using high throughput qualitative (or semi-quantitative) methods, e.g. immunoassays. In case of a presumptively positive result (i.e. including the possibility of a false-positive), it is necessary to perform a second analytical step of confirmation. For that purpose, a highly accurate, quantitative procedure is required, based most often onto physical-chemical technologies. Many analytical methods have been used for the confirmation of targeted compounds as well as for the so-called generic screening (i.e. broad range search of any possible drug or toxicant), but those currently adopted include GC or HPLC with MS or MS^n detection or, more recently, with high resolution MS. In this context, since the ‘90s, CE has started to be considered as an alternative technique, finding a quite large success in the academic research, but so far only limited application in the “real world” [50]. However, a general evaluation of the present real situation leads to conclude that the application of CE in forensic analysis and particularly in forensic toxicology is still limited to only few centres worldwide. This difficulty in penetration into this quite conservative area can be explained also with a scarce enthusiasm of the forensic analysts to use a separation technique based on electrophoretic principles, which they are little confident in. On these grounds, one should point out the importance of the dissemination of the “electrophoretic knowledge” in the field of forensic analysis, which could be preliminary to the use of CE.

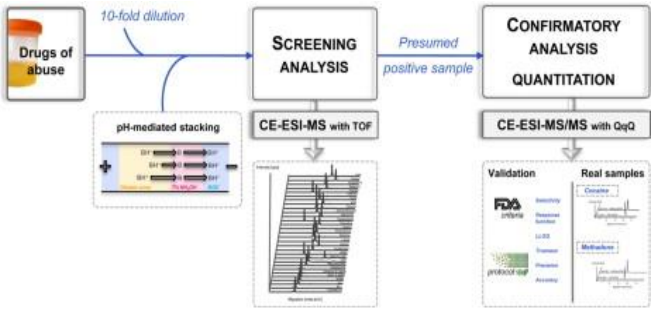


Figure 13. Scheme of two-step forensic toxicology analysis [25].

6. KETAMINE

6.1 INTRODUCTION

Ketamine, a phencyclidine analogue, is a dissociative anaesthetic that, in addition to analgesia, induces loss of consciousness, amnesia and immobility. First produced as Ketalar® in 1962, it was used to treat American soldiers during the Vietnam war and released for civilian use in 1970 [51]. It has been abused since the 1980s for its stimulant, dissociative and hallucinogenic effects particularly by the young generations, therefore its monitoring is valuable for traffic and workplace safety. It is nowadays still used in paediatric emergency retrieval and in veterinary surgery, because of a reduced tendency to give respiratory depression. In fact, its main advantage is to induce profound analgesia and amnesia, while maintaining the cardiopulmonary functions and the protective airway reflexes stable. The main mechanism of action of ketamine on the central nervous system (CNS) occurs as a non-competitive antagonism on the *N*-methyl-D-aspartate (NMDA) receptor Ca^{2+} channel pore. The NMDA channel block, together with the reduction of presynaptic release of glutamate, appear to be the primary mechanisms of the anaesthetic and analgesic actions of ketamine. There is also evidence that ketamine is capable of interacting with opioid receptors and, specifically with μ and κ. Furthermore it acts as an antagonist in the interaction with monoaminergic, muscarinic and nicotinic receptors [40]. Ketamine undergoes extensive hepatic first-pass metabolism to produce various free and glucorinated hydroxylated derivatives [52]. However, its main metabolic pathway occurs trough *N*-demethylation to norketamine, which appears to have 20-30 % activity of its parent drug.

Starting from the 1980s, ketamine began to be a widespread drug of abuse in many countries and, quite recently, also in Italy. Providing with hallucinogenic effects at sub anaesthetic doses, it is often abused to achieve an out-of-body experience by entering the so-called "*K-hole*". Because of these desired effects, ketamine appears particularly dangerous with regards to traffic and workplace safety.

Being a chiral drug, ketamine exists in the form of a racemic mixture of its *S*- and *R*- enantiomers (Figure 14). Interestingly, this drug belongs to the infrequent class of chiral compounds for which the sign of rotation changes when the free base is mutated into the hydrochloride salt with preservation of the absolute configuration [53].

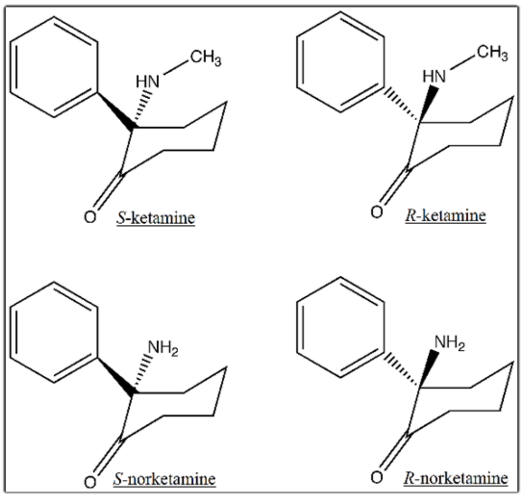


Figure 14. Structures of the enantiomers of ketamine and norketamine.

The *S*- enantiomer of ketamine recently received peculiar attention since it showed a different pharmacodynamic behaviour, when compared to the *R*- enantiomer and/or to the racemate. It proved a four times higher affinity for the phencyclidine site of the NMDA receptor, as well as a greater potency when compared to *R*-ketamine and to the racemic mixture [40,54]. Since lower doses of the *S*-enantiomer are satisfactory to maintain an equivalent state of anaesthesia, fewer side effects and shorter recovery times are expected with the single enantiomer preparation [55,8,56-59]. For these reasons, *S*- ketamine has been released in some countries as an alternative to the racemic ketamine preparations. The different pharmacodynamic behaviour of the two enantiomers implies the need for a chiral analysis method that, to the best of our knowledge, until now, has not been reported in hair samples, particularly including both parent drug and major metabolite. Currently, GC or LC coupled to MS are the techniques of choice for the determination of ketamine, its main metabolite norketamine and, at times, dehydronorketamine in urine and blood samples. The reported LODs (limit of detection) range from 0.02 to 19.00 ng/mL [60-74]. Also, in cases where hair was the biological matrix of interest, GC or LC coupled to MS was the analytical approach of choice with LODs ranging from 2.0 pg/mg to 0.5 ng/mg [75-83]. However, no one of the available analytical methods is enantioselective, showing a clear lack of methodologies.

Because of the availability in the market of both racemic ketamine and its *S*-enantiomer, the chiral analysis herein presented may reveal the type of drug taken by an individual. On the other hand, the chiral analysis of ketamine could also disclose an enantioselective metabolism of the drug.

Consequently, the aim of the present work was to develop an enantioselective CZE method for the separation of ketamine and its major metabolite, norketamine and to validate its application in hair samples.

6.2 MATERIALS AND METHODS

6.2.1 MATERIALS

All used chemicals were of analytical grade. Racemic ketamine HCl and racemic norketamine HCl solutions in methanol (1 mg/mL, 3.66 mM) were purchased from Cerilliant (Round Rock, USA). *S*- and *R*- ketamine, *S*- and *R*- norketamine were kindly provided by the Clinical Pharmacology Laboratory of the University of Bern (Bern, Switzerland). Lamotrigine (used as internal standard, IS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris was purchased from Amersham Biosciences (Uppsala, Sweden) and H_3PO_4 (85%) was from Sigma Aldrich (Steinheim, Germany). HCl 37%, NaOH and n-Hexane were from Carlo Erba Reagents (Milano, Italy). Ethyl acetate was obtained from VWR International S.A.S. (Fontenay-sous-Bois, France). Highly Sulphated-γ-Cyclodextrins (HS-γ-CDs) 20% (w/v) aqueous solution was kindly donated from Beckman Coulter Inc. (Brea, CA, USA). Milli-Q water produced from Millipore Ultra-Pure Water system (Millipore, Bedford, MA, USA) was used for preparation of the buffers and all aqueous solutions.

6.2.2 CE SYSTEM AND SOFTWARE

All CE experiments were performed using a P/ACE MDQ electrophoresis apparatus equipped with UV detection (Beckman Coulter Inc., Brea, CA, USA). The wavelength of detection was set at 200 nm. An uncoated fused-silica capillary of 50 μm i.d. and 45 cm total length (35 cm to the detection window) was utilised.

The capillary was washed each day of use before analysis runs with 1 M NaOH, 0.1 M NaOH, water, running buffer at 20 psi for 5 min for each solution. Prior to each run, the capillary was rinsed with water, followed by running buffer at 20 psi for 1 min each. The capillary cartridge temperature was maintained at 20 °C. Separation occurred at a voltage of 20 kV, in 15 mM Tris phosphate (pH 2.5) containing 0.1% HS-γ-CDs (w/v). Data were collected and analysed by using MDQ 32 Karat software from Beckman Coulter.

6.3 PREPARATION OF STANDARD SOLUTIONS

Ketamine and norketamine stock solutions were prepared in methanol at a concentration of 0.5 mg/mL (1.83 mM) of each enantiomer; the working standard solutions were prepared in water at concentrations ranging from 5 µg/mL to 1 ng/mL from the stock solutions. Lamotrigine (IS) stock solution was prepared in methanol at a concentration of 1 mg/mL; the working standard solutions were prepared in water at concentrations ranging from 5 µg/mL to 100 ng/mL. All solutions were stored at -20 °C until analysis.

6.4 SPECIMENS

Drug-fortified hair samples were obtained by spiking blank hair (collected from eight healthy volunteers that had never used either the target drug nor any other illicit drugs) with appropriate standard solutions. Real-world hair samples (n=12) were also analysed. These samples had been previously collected from subjects whose driving license had been confiscated for drug abuse and who were found “positive” for ketamine at a hair toxicological screening test using a validated UHPLC-QqQ MS non-chiral method (ketamine concentrations ranging from 330 pg/mg to 107 ng/mg).

6.5 SAMPLE PREPARATION PROCEDURE

Hair samples were first washed with different solvents as follows: 1% SDS for 15 min, water for 5 min, and finally with methanol for 5 min. Hair was allowed to air dry at room temperature under a fume hood prior to the final washing step with methanol. The purpose of washing hair samples was to remove any potential external contaminant present on the surface. The washed and dried hair (50 mg) was then cut into small sections of ~1-3 mm and mixed with 500 μL of 0.1 M HCl. At this stage, lamotrigine (IS) and, for drug-fortified hair, ketamine solutions were added. Then, the mixture was sonicated for 1 hour and incubated overnight in water bath at 37° C. On the following day, the liquid portion was separated from the hair and pipetted into new test tubes. The aqueous phase was brought to pH ~10 with 200 μL of 1 M NaOH and added with 3 mL of hexane/ethyl acetate mixture (50:50 v/v). The resulting mixture was shaken for 4 min and centrifuged for 10 min at 2,500 *X* g. The organic phase (2.5 mL) was separated from the aqueous layer and transferred into a new glass tube, where it was acidified by addition of 10 μL of 10 mM HCl and evaporated at 37 °C under nitrogen flow. The dried extract was reconstituted with 250 μL of water and 5 μL of 10 mM HCl for CE analysis.

6.6 SAMPLE INJECTION AND SAMPLE STACKING

Following the rinsing process described above, prior to each run, a plug consisting of 15 mM Tris phosphate (pH 2.5) was hydrodynamically injected (0.5 psi, 40 s) in order to create a zone of lower conductivity than the running buffer where also the negatively charged cyclodextrins were present. The sample was subsequently electrokinetically injected (7 kV, 20 s). After sample injection, the inlet and outlet reservoirs were replaced with 15 mM Tris phosphate (pH 2.5) containing 0.1% of HS - γ- CDs 20% (w/v) and a “normal polarity” of 20 kV was applied for the electrophoretic run.

6.7 METHOD VALIDATION

The following parameters were studied: specificity, linearity, LOD, LOQ, extraction recovery, matrix effect, precision and accuracy.

6.8 RESULTS AND DISCUSSION

6.8.1 OPTIMISATION OF CE CONDITIONS

In order to optimise the separation buffer, different concentrations and pH values of Tris, KH_2PO_4 , $NH_4H_2PO_4$ and triethylammonium phosphate buffers were tested. Moreover, different classes of CDs were evaluated as chiral selectors, including native β-CD and its derivatives, such as DM-β-CD, HP-β-CD, S-β-CD, CM-β-CD, as well as native γ-CD and its highly sulphated (HS) derivatives. The optimised running buffer, modified from a published one [40], was composed of 15 mM Tris adjusted to pH 2.5 with diluted phosphoric acid and 0.1% HS-*γ*-CD. Using this running buffer, the ketamine and norketamine enantiomers were separated in less than 10 minutes. Under the conditions described in 6.2.2, a resolution (USP) of 2.0 for NK enantiomers, and 2.4 for K enantiomers was achieved.

6.8.2 SAMPLE STACKING

Being hair a peculiar biological matrix, which, because of aesthetical reasons, can be collected only in small amounts, sensitivity was a crucial feature of the analytical method. Consequently, with the aim of reaching a suitable sensitivity, a Field Amplified Sample Stacking (FASS) technique was included in the injection procedure. Under optimised conditions, FASS enhanced analytical sensitivity by a factor of 1,000, when compared to hydrodynamic injection (1 psi, 5 s) (see Figure 15).

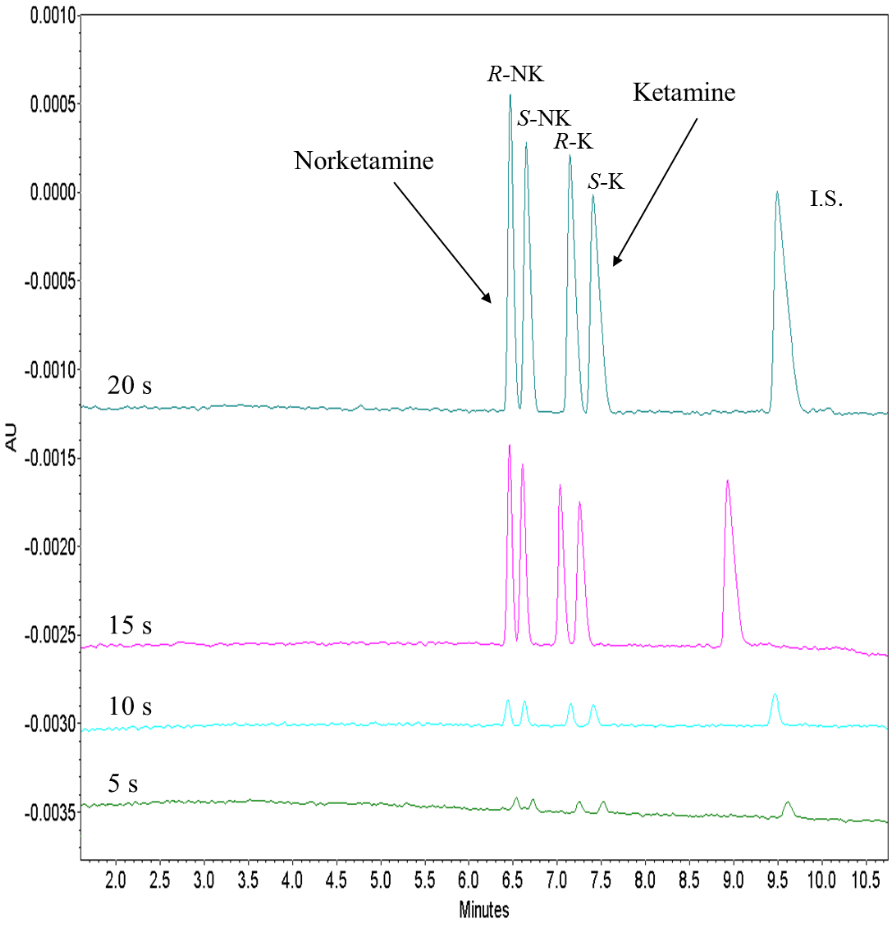


Figure 15. FASS injection of racemic ketamine (K) and norketamine (NK) added to a blank hair extract. For conditions, see section 6.6. Concentration of each enantiomer: 125 ng/mL.

6.8.3 SAMPLE PREPARATION

Several sample preparation procedures for ketamine extraction from human hair are available in the literature [77,79,82,83], from which we selected the simplest two [83,79] as a starting point to develop an original methodology. In short, a liquid-liquid procedure was chosen, as described in section 6.5, as less time consuming, easier to be transferred to other labs and cheaper than SPE. As in any liquid-liquid extraction, pH control and the extraction solvents played a crucial role for the optimisation of recovery. On the basis of the pKa of ketamine (7.5) and considering the acidity of the hair incubation mixture (500 μL of 0.1 M HCl), sample alkalinisation was essential for solvent extraction. This point was tested using different concentrations and volumes of NaOH solution. The highest recovery of analytes was achieved with 200 μL of 1.0 M NaOH. The candidate solvents for extraction were ethyl acetate, hexane, dichloromethane and mixtures of them. The highest extraction efficiency was achieved using a 1:1 hexane/ethyl acetate mixture (Table 2), being those of the other solvents 2-3 times lower.

6.8.4 METHOD VALIDATION

A validation process of the analytical method, including specificity, LOD, LOQ, biological matrix effect, recovery, precision, accuracy and linearity, was carried out according to the requirements of forensic toxicology [84,85]. The specificity was determined by analysing eight hair samples (blanks) from well known laboratory personnel who declared no use of any drugs in the last 6 months (Figure 16).

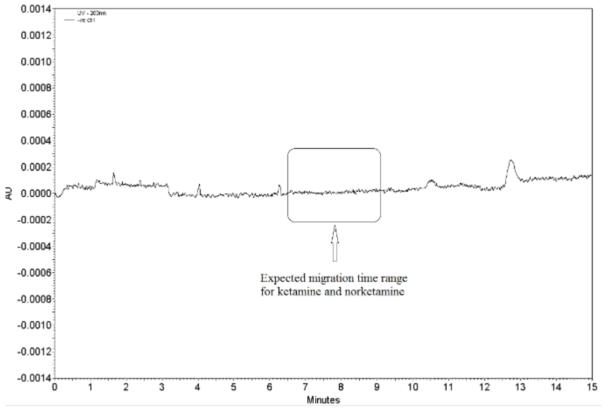


Figure 16. Example of extraction from blank hair sample.

Specificity was judged satisfactory if the signals relative to endogenous compounds did not interfere with those of the analytes of interest. Peaks that showed an S/N value lower than the LOD at the migration times of the four analytes (ketamine and norketamine enantiomers) were not considered as interferences.

LOD and LOQ were estimated in spiked hair samples at signal-to-noise ratios of three and ten, respectively. The values obtained were 0.08 ng/mg for the LOD and 0.25 ng/mg for the LOQ for each enantiomer. In order to evaluate the quantitative performances of the method, five different concentrations of analytes were used to spike blank hair samples. These five concentrations were tested in quintuplet over the range of racemic ketamine and norketamine concentrations 0.5-8.0 ng/mg, using the I.S. at 1 ng/mg. Linearity between the normalised peak-area ratios of each enantiomer (y) to the I.S. and the concentration (x, ng/mg) of the analytes in the spiked hair samples was studied (Figure 17). Linearity equations and the related correlation coefficients are reported in Table 1.

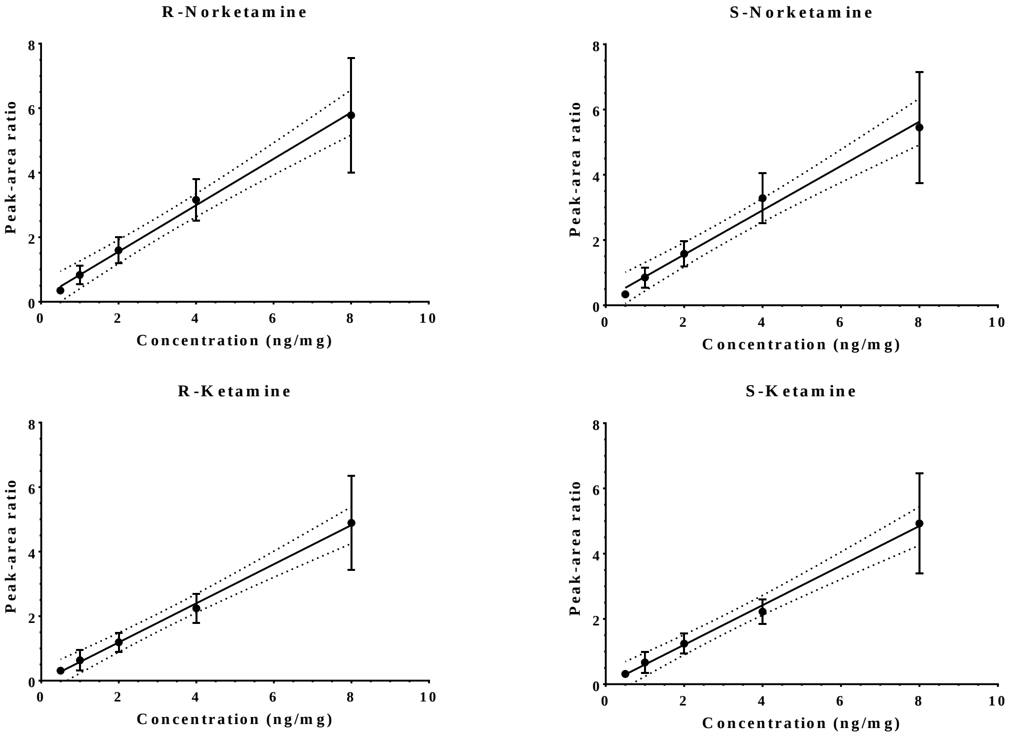


Figure 17. Calibration curves obtained from spiked hair samples. 95% confidence intervals were calculated.

Table 1. Regression analysis for the determination of the enantiomers of ketamine and norketamine.

Enantiomer	Regression equation	Correlation coefficient (R^2)
R-norketamine	$y^a = (0.72 \pm 0.04)x^b + (0.12 \pm 0.05)$	0.997
S-norketamine	$y^a = (0.68 \pm 0.02)\, x^b + (0.20 \pm 0.11)$	0.988
R-ketamine	$y^a = (0.61 \pm 0.09)\, x^b - (0.02 \pm 0.08)$	0.998
S-ketamine	$y^a = (0.61 \pm 0.11)\, x^b - (0.01 \pm 0.05)$	0.996

Concentration range: 0.5-8.0 ng/mg, samples spiked with racemic standard solutions (n=5).

[a] *Normalised peak-area ratios of the related enantiomer to the I.S.*

[b] *Concentration of the analyte (ng/mg).*

The biological matrix effects and extraction recoveries were evaluated at three concentrations of each enantiomer of ketamine and norketamine [i.e. low (0.13

ng/mg), medium (0.50 ng/mg), and high (2.00 ng/mg), equivalent to the addition of standard solutions to obtain final concentrations of 13 ng/mL, 50 ng/mL and 200 ng/mL respectively], following [86]. Three sample sets were prepared at such concentrations: standard solutions of ketamine and norketamine (set A), hair spiked after extraction (set B), and hair spiked before extraction (set C). The matrix effect was estimated by comparing the quantitative results from set A and set B. The extraction recovery was calculated by comparing the quantitative results from set A and set C. All values, expressed as percentages, are reported in Table 2.

Table 2. Extraction recovery and matrix effect.

Enantiomer	Expected amounts in ng/mg (set A)[a]	Amount measured in set B (ng/mg)[b]	Matrix effect (%) n=5	Amount measured in set C (ng/mg)[c]	Extraction recovery (%)*n=5*
R-norketamine	0.13	0.10 (±0.03)	77	0.10 (±0.02)	79
	0.50	0.34 (±0.04)	68	0.32 (±0.03)	64
	2.00	1.27 (±0.15)	64	1.47 (±0.09)	74
S-norketamine	0.13	0.11 (±0.04)	89	0.11 (±0.03)	91
	0.50	0.33 (±0.03)	66	0.32 (±0.03)	64
	2.00	1.27 (±0.15)	64	1.50 (±0.08)	75
R-ketamine	0.13	0.14 (±0.08)	112	0.09 (±0.03)	73
	0.50	0.38 (±0.06)	76	0.25 (±0.04)	49
	2.00	1.26 (±0.17)	63	1.09 (±0.14)	54
S-ketamine	0.13	0.15 (±0.09)	119	0.11 (±0.04)	89
	0.50	0.39 (±0.06)	79	0.25 (±0.04)	51
	2.00	1.31 (±0.18)	66	1.05 (±0.15)	53

Determined from the assay of blank hair samples spiked with standard solutions (n=5).
[a] *Peak area ratios expected by standard solutions.*
[b] *Comparison of peak area ratios obtained by extracts spiked after extraction vs. standard solutions.*
[c] *Comparison of peak area ratios obtained by extracts spiked before extraction vs. standard solutions.*

Intra- and inter- assay precision [expressed as relative standard deviation (%RSD)] was assessed by extracting and analysing six replicates of hair (n=6) spiked with a mixture of each enantiomer of ketamine and norketamine at 0.25 and 1.00 ng/mg on six consecutive days. Precision was evaluated on both peak areas and migration times (MT). Accuracy was determined as relative error (%RE) vs. the expected concentration on the same samples. Precision and accuracy values, expressed ad %RSD and %RE are reported in Table 3.

Table 3. Intra- and inter- day variability. Precision and accuracy are expressed as average area (SD) n=6.

Enantiomer	Added amount (ng/mg)	Measured amount (ng/mg)	Area Precision (%RSD)		Area Accuracy (%RE)[a]		MT[b] Precision (%RSD)	
			Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
R-norketamine	0.25	0.24 (± 0.03)	11.6	14.0	-16.4	-16.6	0.99	0.24
	1.00	1.00 (± 0.20)	14.5	14.7	-14.6	-17.4	0.75	0.21
S-norketamine	0.25	0.25 (± 0.03)	11.4	14.6	-17.0	-17.2	1.02	0.25
	1.00	1.00 (± 0.20)	14.3	14.8	-13.8	-15.9	0.78	0.22
R-ketamine	0.25	0.23 (± 0.06)	12.9	14.4	-22.6	-22.8	1.14	0.31
	1.00	1.07 (± 0.20)	13.4	12.7	-17.7	-21.3	0.85	0.28
S-ketamine	0.25	0.25 (± 0.06)	14.4	17.9	-22.3	-22.9	1.17	0.34
	1.00	1.10 (± 0.21)	15.4	15.7	-22.7	-29.2	0.88	0.30

[a] RE (% relative error) = (area measured – area expected/) x 100/(area expected).
[b] MT (migration time)

6.8.5 ANALYSIS OF HAIR SAMPLES FROM KETAMINE ABUSERS

Twelve hair samples from ketamine abusers were obtained from subjects to whom the driving license had been suspended/withdrawn for "driving under the influence" of drugs of abuse in years 2008 – 2013 in the province of Verona and Vicenza. These samples had previously been screened for the most common drugs of abuse by LC-MS, showing the presence of ketamine. Hair samples were washed, extracted and analysed according to the procedures described in section 6.5. Notwithstanding the presence of unknown peaks, signals referred to the ketamine and norketamine enantiomers on the basis of the relative migration time (ratio by IS) and UV spectrum were clearly identified (Figure 18).

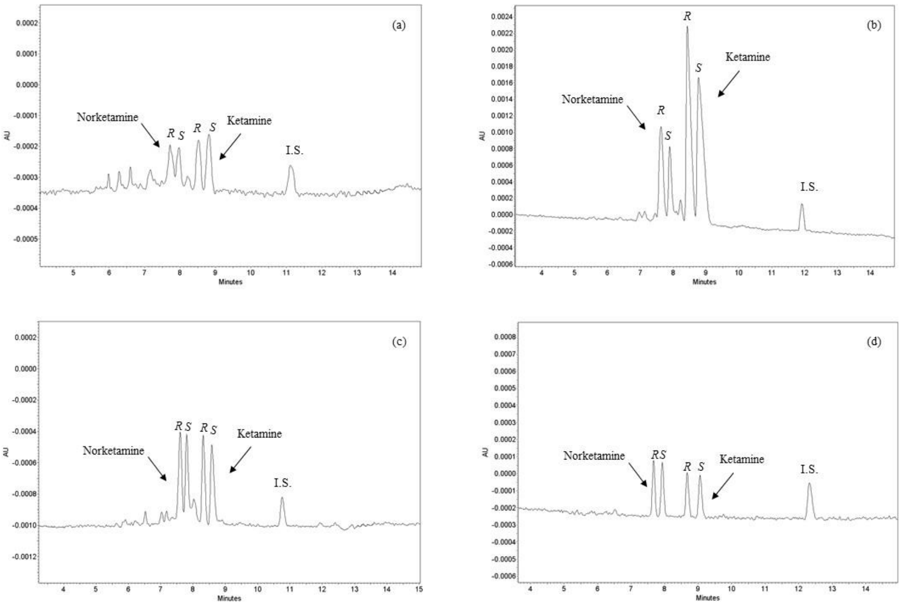


Figure 18. Examples of 3 real-world samples (a-c), extracted according to procedure described in 6.5, against (d) a standard solution containing ketamine, norketamine and lamotrigine (I.S.).

6.9 CONCLUSIONS

Hair analysis has become an important tool in forensic toxicology for the understanding of the drug abuse history of individuals, offering a much broader “time window” compared to the more traditional blood and urine analyses. Being nowadays ketamine an emerging drug of abuse as a racemic substance, but, possibly, also as single *S*-enantiomer, simple and low cost chiral analysis methods are needed. The experimental work herein discussed was aimed at achieving the enantioselective determination of ketamine in hair, as well as, for the first time, of its main metabolite, norketamine. The optimised analytical conditions offered excellent separation power and reproducibility in terms of migration times, extraction recovery, and linearity. Electrokinetic injection allowed for the detection of ketamine and norketamine enantiomers at trace levels in hair samples with a LOD of 0.08 ng/mg in a short analysis time (10 min). The validated method was also preliminarily tested with samples collected from abusers, which had been previously tested with a LC-MS screening method validated for drugs of abuse on hair specimens. The method is also suitable for studying a possible enantioselective metabolism of the drug.

7. BACLOFEN

7.1 INTRODUCTION

Baclofen (4-amino-3-*p*-chlorophenylbutyric acid) is a *p*-chlorophenyl analogue of *γ*-amino-butyric acid (GABA) (Figure 19). GABA, a neutral amino acid, is an inhibitory neurotransmitter involved in the control of neuronal activity in the mammalian central nervous system and in the regulation of many physiological mechanisms [87]. It has been proved that the mechanism of action of GABA occurs at least through two different receptor sites, namely $GABA_A$ and $GABA_B$ with different binding properties [88].

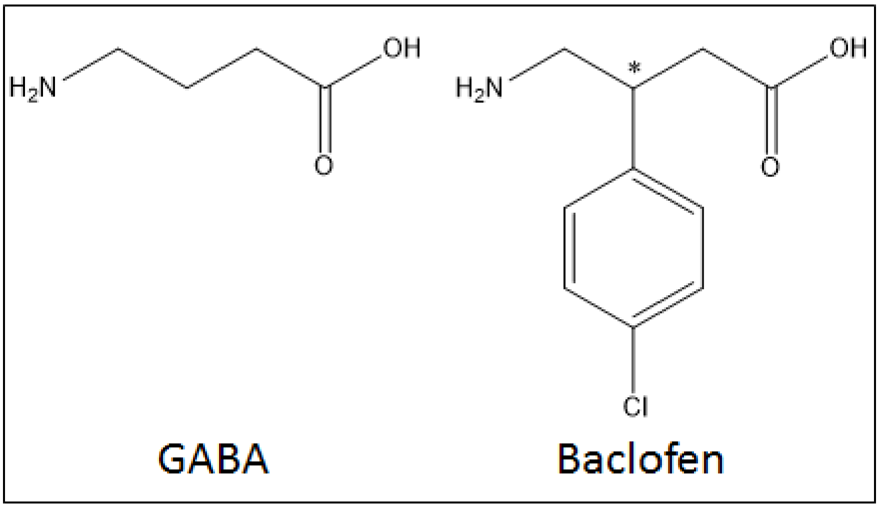


*Figure 19. Chemical structures of GABA and baclofen. * = chiral centre of baclofen.*

Baclofen is one of the selective agonists for the $GABA_B$ receptor and is commercially available under the names of Baclofene-Irex® and Lioresal®. Since its introduction for therapy in 1967 as a skeletal muscle relaxant, it has been widely used in the treatment of multiple sclerosis or spasticity caused by spinal or cerebral injury. However, baclofen has recently attracted considerable attention as a potential medication for alcoholism. In fact, being an agonist for the $GABA_B$ receptor, it allows the decrease of the severe symptoms of withdrawal from alcohol abuse (tremors, confusion, *delirium tremens*) and it proved effective for subjects with drug-seeking behaviour. In particular, in France, baclofen is recently being prescribed to alcohol-dependent patients by administering high doses with the aim of managing alcohol dependence despite the absence of high-level evidence.

Concerning the chemical structure, the *p*-chlorophenyl substituent of baclofen makes it a chiral molecule with the two optical isomers (Figure 20) differing in their pharmacodynamic and toxicological properties. It has been shown that the *R*-(-)-enantiomer is about 100 times more active than the *S*-(+)-enantiomer [89] and therefore *R*-baclofen is much more toxic than *S*-baclofen. Besides, the "ineffective" *S*-enantiomer antagonizes the action of the effective *R*-enantiomer, so that *R*-baclofen is also substantially more effective than the racemate [90].

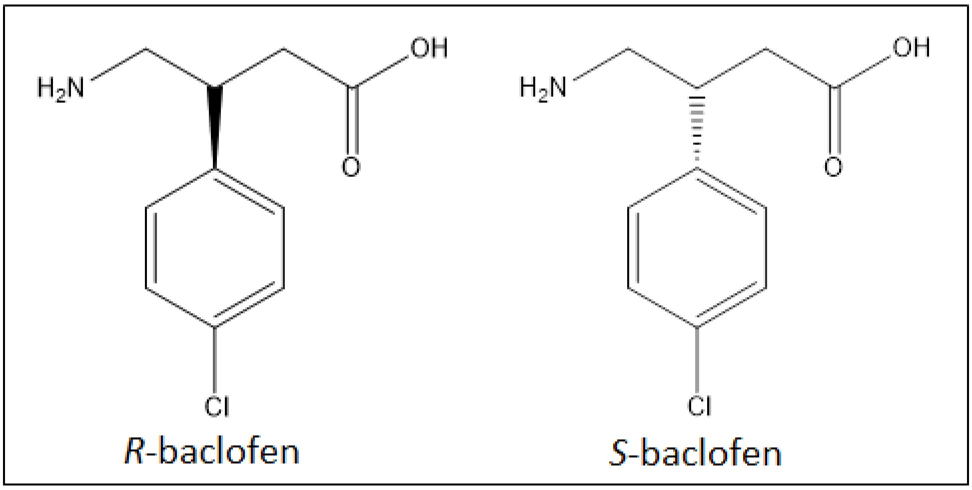


Figure 20. Structures of the enantiomers of baclofen.

Nevertheless, baclofen is clinically administered as a racemic mixture both in the treatment of spasticity and in the withdrawal from alcoholism. Despite the administration of racemic baclofen for all groups of patients, the kinetic disposition of the two enantiomers may be different; hence, the investigation of the pharmacokinetic behaviour of both enantiomers is often desirable.

Since chiral separation by CE has been widely discussed in the previous chapters, it is worthwhile introducing the basic principle of enantiomeric resolution by liquid chromatography (LC), which may be divided into two categories. One is the enantioseparation by direct approach (without involving a covalent linkage with any chiral selectand); it can involve the use of chiral stationary phases (CSPs), chiral mobile phase additives or a chiral selector. A number of chiral resolutions have been achieved by direct methods that employ CSP columns containing immobilised chiral selectors. Direct enantioseparation could also be achieved by chiral ligand-exchange chromatography, where a chelate formation between the enantiomers of the racemate analyte and the metal–*L*-amino acid complex occurs. This strategy has mostly been applied to the resolution of a limited number of racemic substances, such as amino acids but not pharmaceutical formulations.

The indirect approach, on the other hand, involves the reaction of the racemic analyte with a suitable chiral derivatisation reagent (CDR), prior to LC separation step through the formation of a covalently bonded diastereomeric pair.

To date, racemic baclofen has been resolved in various biological matrices (mainly plasma and urine) using techniques based on gas or liquid chromatography, HPLC coupled to UV–vis, MS and fluorescent detection, HPLC with fluorescence detection after derivatisation and HPLC with electrochemical detection, as well as CE and CE-MS. Besides, enantiomers of baclofen have been resolved in pharmaceutical formulations using enantioselective membrane electrodes based upon maltodextrin and α- and β-cyclodextrins [91].

Taking into account the medicinal importance and clinical applications of baclofen (now also as a potential treatment for alcoholism, as mentioned above) along with its characteristic chemical and structural properties, it was considered worthwhile to develop a sensitive and efficient enantioseparation strategy. On these grounds, the aim of the present study was to improve existing methods in CE in order to obtain a highly efficient, cost effective and simple technique intended to resolve racemic baclofen in biological matrices.

7.2 MATERIALS AND METHODS

All used chemicals were of analytical grade. Racemic baclofen was obtained by the pharmacy of *Policlinico G. B. Rossi* Hospital in the form of Lioresal® (10 mg in 20 mL solution for infusion, i.e. 500 μg/mL) produced by Novartis (Basel, Switzerland). Tris was purchased from Amersham Biosciences (Uppsala, Sweden). H_3PO_4 (85%), Poly-(ethylene glycol) (PEO), ethylene glycol, methanol, 2-hydroxypropyl- β-CD (HP- β-CD), sulphated- β-CD (S-β-CD) and β-CD were obtained from Sigma Aldrich (Steinheim, Germany). A second lot of β-CD (in-house synthesised) were kindly donated from the National Research Council (CNR, Rome, Italy). Na_2HPO_4 and ethanolamine were purchased from Merck (Darmstadt, Germany). Acetonitrile (ACN) and NaOH were obtained from Carlo Erba Reagents (Milano, Italy). Highly sulphated-γ-CDs (HS - γ- CDs) 20% (w/v) aqueous solution was kindly donated from Beckman Coulter Inc. (Brea, CA, USA). Milli-Q water, produced from Millipore Ultra-Pure Water system (Millipore, Bedford, MA, USA) was used for preparation of the buffers and all aqueous solutions.

7.2.1 CE SYSTEM AND SOFTWARE

CE experiments were performed using two different electrophoresis apparatuses: *i.* P/ACE MDQ equipped with UV detection (Beckman Coulter Inc., Brea, CA, USA) and *ii.* Agilent 7100 Capillary Electrophoresis System (Agilent Technologies, Santa Clara, CA, USA). Three different wavelengths of detection were tested by switching from 196, 200 to 214 nm. Uncoated fused-silica capillaries of 50 μm i.d. and 45 cm total length (35 cm to the detection window) were utilised. The capillary cartridge temperature was maintained at 20 °C. Data were collected and analysed by using MDQ 32 Karat software from Beckman Coulter and Agilent Lab Advisor Software from Agilent Technologies, respectively.

7.2.2 PREPARATION OF STANDARD SOLUTIONS

Baclofen stock solutions were prepared from the Lioresal® solution of 500 μg/mL in distilled water at a concentration of 50 μg/mL of racemic mixture; the working standard solutions were prepared in water from the stock solution. All solutions were stored at -20 °C until analysis.

7.3 RESULTS AND DISCUSSION

7.3.1 OPTIMISATION OF CE CONDITIONS

There are many factors to take into account in order to optimise the separation buffer with the final aim of obtaining the chiral resolution of two enantiomers in CE:

- Buffer composition, hence its ionic strength and pH value;
- Buffer concentration;
- The effect of organic modifiers on the buffer;
- Coating of the capillary where the separation occurs;
- Enhancing additive, in the form of a chiral selector;
- Optimal separation conditions, namely temperature, voltage, and polarity;
- Increase in sensitivity (sample stacking strategies).

In the present study, different buffer compositions were tested in order to obtain, at first, the resolution of baclofen enantiomers from its racemate. The first attempt was performed by using the same buffer composition used for the resolution of ketamine and norketamine and previously described (15 mM Tris at a pH of 2.5 reached by addition of diluted H_3PO_4 and added with 0.1% HS-γ-CD 20 % (w/v)). By using this running buffer, baclofen was injected *via* both hydrodynamic injection (1 psi 5 s) and electrokinetic injection developed for ketamine and norketamine described in section 6.6. Under these conditions, neither hydrodynamic injection nor electrokinetic injection provided the resolution of the *R*- and *S*- enantiomers. The concentration of CDs was increased from 0.1 % of the 20 % stock (w/v) to 1, 3 and 5 % yet a single peak (racemic baclofen) migrated in less than 10 minutes.

Following a published method for baclofen resolution [92], 50 mM Na_2HPO_4/ACN (95:5) at neutral pH (reached by H_3PO_4 85 %) and 10 mM β-CDs synthesised in-house was employed for the electrophoretic separation, nevertheless, no resolution of the two enantiomers occurred. β-CDs purchased from Sigma Aldrich were, in a second step, added to the phosphate buffer and tested with no success. Different concentrations of such CDs, ranging from 5 mM to 20 mM, did not provoke the desired resolution whichever. The described conditions were moreover tested at different separation voltages (5, 15, 25, and 30 kV) and through two different wavelengths (200 and 214 nm), all providing with a single peak for racemic baclofen. In addition, ACN, which served as an organic modifier for the buffer, was added to the solution at concentrations of 5 % and up to 30 % and, secondly, was substituted with MeOH. MeOH proved to cause peak broadening already at a percentage of 5 % and was, therefore, excluded from further investigation. Finally, all the conditions hereby described were performed with different concentrations of a second class of CDs, namely 2-HP-β-CD (2-hydroxypropyl-β-CD). Hitherto 10, 20 and 30 mM (with 30 mM being the maximum concentration for solubility) were not sufficient to resolve racemic baclofen.

The third strategy was based on a validated method [41], which was hereby modified according to the following described stages. Firstly, a running buffer composed of 25 mM H_3PO_4 at pH 2.5 (reached by addition of ethanolamine) and HS-γ-CDs was employed to determine the migration time of baclofen and, potentially, resolve its optical isomers. HS-γ-CDs were added to the running buffer at percentages ranging from 0.6 to 3 % with no success in terms of resolution. Besides, no dynamic coating (in the name of PEO as per [41]) was applied to the separation compartment at this stage. Subsequently, ethylene glycol was employed for the said dynamic coating, yet a single peak migrated as racemic baclofen.

Finally, 0.4 % PEO was employed in order to obtain a dynamic coating with the following rinsing program to be performed every day prior to electrophoretic separations:

- 0.1 M NaOH, 20 psi x 5 min
- H_2O, 20 psi x 1 min
- 0.4 % PEO, 25 psi x 1 min
- H_2O, 25 psi x 1 min
- Running buffer, 25 psi x 3 min

With such dynamic coating of the capillary, the optimal running buffer proved to be composed of 25 mM H_3PO_4 at pH 2.5 (reached by addition of ethanolamine) and 1 % HS-γ-CDs (w/v of 20% CDs). Concerning the injection, racemic baclofen was hydrodynamically injected at 0.5 psi x 5 s. Being the HS-γ-CDs negatively charged, reverse polarity was applied at -20 kV and with a wavelength set at 200 nm. In such conditions, baclofen was resolved into its *R*- and *S*- enantiomers with a resolution of 1.12 in about 20 minutes (Figure 21).

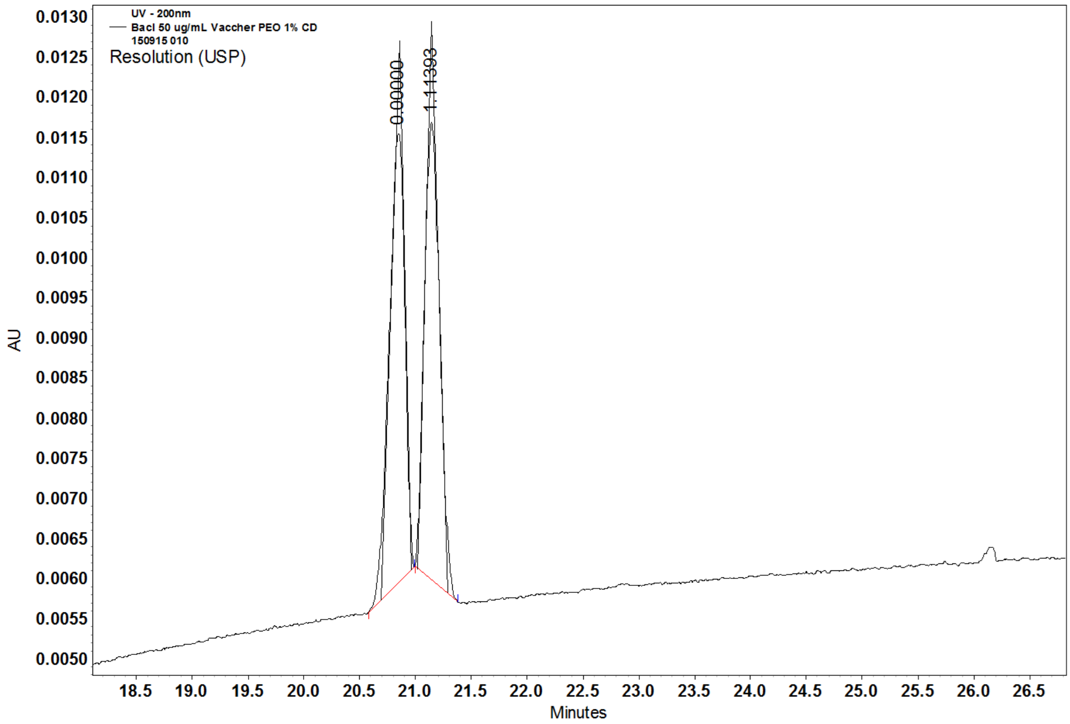


Figure 21. Resolution of baclofen enantiomers. Conditions: running buffer composed of 25 mM H_3PO_4 *pH 2.5 and 1 % HS-γ-CDs (w/v); hydrodynamic injection (0.5 psi x 5 s); reverse polarity = -20 kV; 200 nm. R = 1.12.*

Nevertheless, such resolution appeared “low” in view of the need of an extra step of sample stacking, which inherently could damage the enantioseparation. Therefore, a substitution of chiral selectors was made to such optimised conditions: 5 % (w/v) S-β-CDs provided a higher resolution (R=2.79) with a decrease in the migration time, occurring in less than 10 minutes (Figure 22).

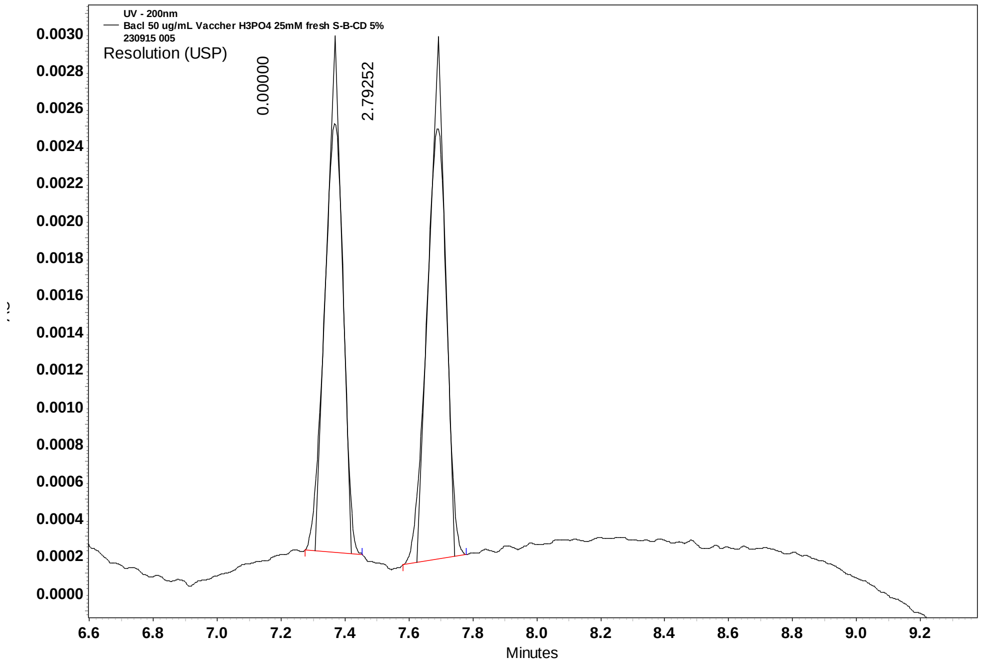


Figure 22. Increase in resolution of baclofen enantiomers (R = 2.79). Conditions described in Figure 21 except for the chiral selector: 5 % S-β-CD (w/v).

As mentioned above, the sensitivity needed to be further improved and consequently the resolution. For these reasons, the method was transferred to another capillary electropherograph (Agilent 7100 Capillary Electrophoresis System) by maintaining the latest separation conditions. A further investigation involved the selected wavelength, since this electropherograph allows to record three set wavelengths simultaneously. Therefore, the wavelengths of 196, 200 and 214 nm were investigated. As a result, the optimal wavelength in terms of peak areas and resolution proved to be 196 nm, with the resolution nearly doubled (R=5.43). Nonetheless, the migration time increased to 15-16 min for both optical isomers (Figure 23).

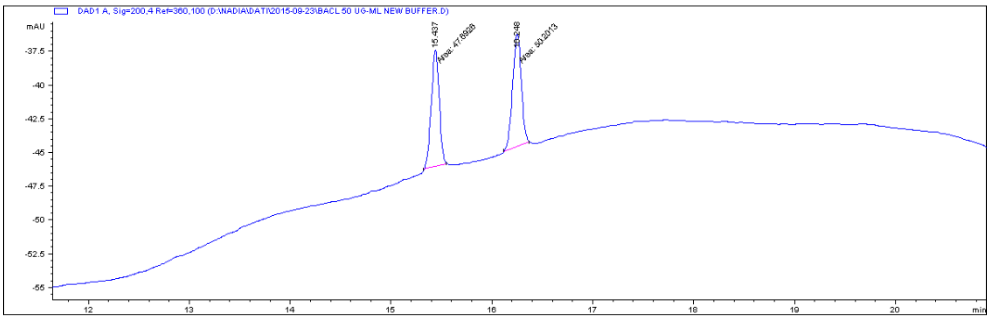


Figure 23. Improved analytical sensitivity (R=5.43) in the enantioseparation of baclofen. Conditions: 25 mM H_3PO_4 pH 2.5 and 5 % β-CDs (w/v); hydrodynamic injection (0.5 psi x 5 s); reverse polarity = -20 kV; 196 nm.

7.3.2 SAMPLE STACKING

At all stages of the method optimisation described above, a significant concentration of baclofen was injected each time (50 µg/ mL) in order to assure a correct visualisation of the peaks. This was related with the low molar absorptivity of the compound, lacking active chromophores. Although baclofen is administered to alcoholic patients in high doses, the use of a sensitive chiral method is needed in order to detect concentrations of baclofen below the pharmaceutical range in plasma (100 ng/mL). Consequently, with the aim of reaching a suitable sensitivity, three different sample-stacking strategies were investigated: FASS, electrokinetic injection (EKI) and large volume injection (LVI). Concerning FASS, a plug consisting of the running buffer excluding the CDs (i.e. 25 mM H_3PO_4 at pH 2.5) was hydrodynamically injected prior to sample injection in order to create a zone of lower conductivity than the zone of the running buffer where also the negatively charged cyclodextrins (5 % (w/v) S-β-CDs) were present. Different lengths of the said plug were injected (1 psi x 5-80 s) with no resulting increase in the peaks areas, although resolution was not affected heavily. Subsequently, the same plug conditions at 1 psi for a time ranging from 5 to 80 s were combined with injection by voltage with different values ranging from 7 to 15 kV (in both polarities) for 20 to 60 s. In these combinations, EKI provided a slight decrease in resolution but no noteworthy increase in peak areas. The most significant enhancement in analytical sensitivity (by a factor of 20), when compared to hydrodynamic injection (0.5 psi, 5 s), was obtained through LVI with a sample injection of 0.5 psi x 70 s (Figure 24).

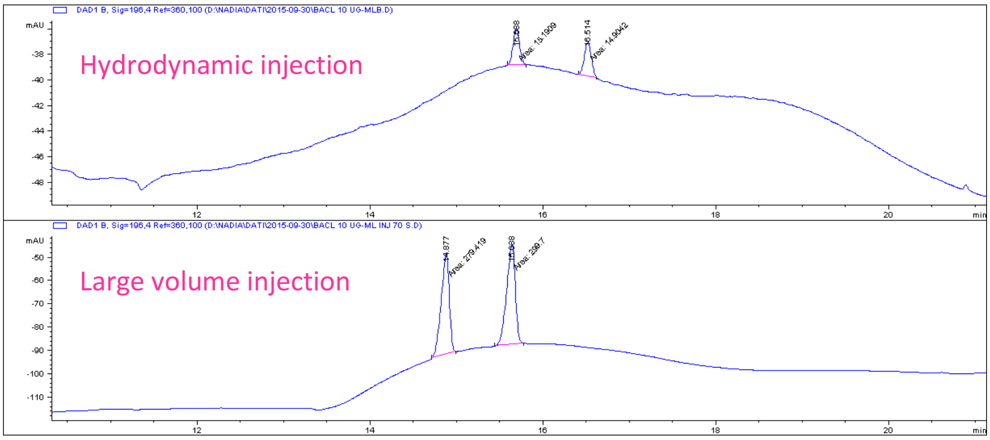


Figure 24. Sample stacking of baclofen enantiomers obtained by LVI (0.5 psi x 70 s). R=3.54, compared to hydrodynamic injection (0.5 psi x 5 s, R=5.43).

Such approach allowed the achievement of a LOD of 500 ng/mL, which is nevertheless “higher” than the desired value of 100 ng/mL. In addition, a loss in resolution was observed (R=3.54), still maintaining the migration times of both enantiomers under 20 min.

7.4 PRELIMINARY CONCLUSIONS AND FUTURE DIRECTIONS

Despite the extensive variety and availability of numerous substances of abuse spread nowadays in many countries, alcohol still persists as the mostly abused substance worldwide. For this reason, it is imperative that medical research continues to discover treatments to redeem from alcoholism, from which it is completely possible to fully recover. Consequently, the understanding of alcohol abuse withdrawal symptoms and their treatment appeared to us as significant as the alcohol abuse investigation itself. Since baclofen proved to decrease the severe symptoms of withdrawal from alcohol abuse and is being prescribed in high doses despite the absence of high-level evidence, devoted analytical methods appear necessary. In particular, being *R*- and *S*- baclofen enantiomers with highly different pharmacokinetic and pharmacodynamic features, it is noteworthy to study their behaviour especially from the analysis of biological matrices. The experimental work herein discussed was aimed at achieving the enantioselective determination of baclofen in plasma. Notwithstanding the analytical conditions were optimised to offer excellent separation power and reproducibility in terms of migration times, the desired analytical sensitivity was not achieved hitherto. In fact, baclofen was successfully resolved in less than 20 min with a resolution up to R=5.5, with the lowest limit of detection being 500 ng/mL (i.e. five times higher than the expected value of 100 ng/mL). On these grounds, it is evident that the main future direction will be to further improve the analytical sensitivity of the method. One possible strategy could be to couple CE to MS to accomplish the needed sensitivity increase. Subsequently, the method will have to be validated according to the requirements of forensic toxicology [84] in order to study a possible enantioselective metabolism of the drug. Only after a full validation process, it will be possible to transfer the technique to biological matrices and to apply the further validated method on real-world samples derived from alcoholic patients treated for alcohol withdrawal syndrome.

8. COUPLING OF CE WITH MS

8.1 INTRODUCTION

Capillary Electrophoresis - Mass Spectrometry (CE-MS), as previously mentioned, couples two analytical techniques based on different chemical-physical principles (for this reason called “orthogonal”), being particularly suitable in forensic analysis, where unequivocal identification and accurate measurement are strictly required.

Among the advantages of CE-MS, it is worth stressing the ability to deal with samples often scarce in quantity, nevertheless requiring preservation for further cross-analysis in disputed cases. In addition, crime scene evidence is in many cases highly contaminated and/or degraded, hence needing to undergo accurate separation in order to get rid of all possible interfering compound preliminarily to the MS identification and quantitation of the substance(s) of interest. Lastly, forensic samples often show high complexity, because of their biological origin, of the presence of metabolites and/or degradation products and potential contaminants.

All of these critical conditions can be tackled and, in most cases, overcome by using CE-MS, which, differently from gas and liquid chromatography coupled to MS, mostly works in open micro-tubular compartments (capillaries) without any packing material.

Last-but-not-least, CE separations are based on physical chemical principles different from those operating in liquid chromatography, thus being “orthogonal” to this more traditional separation technique. This means that the results from the two methodologies, if in agreement, are mutually confirmatory, thus meeting a fundamental requirement of forensic analysis (i.e. confirmation of a result by a different method, analytically orthogonal to the previous one).

The first coupling of CE with MS, reported by Olivares et al. [93] in 1987, consisted of a single quadrupole mass spectrometer coupled to CE. In the following years, a variety of MS systems have been coupled to CE, the most common being quadrupoles and ion traps. The use of such analysers provides low sensibility when operating in the single ion monitoring (SIM) mode, which however focuses the analyser to monitor a single *m/z* ratio, thus sacrificing the detection window. In addition, these analysers suffer from limited scan speed, which is hardly compatible with the extremely narrow peaks, often migrating close to each other, typical of CE analyses. A promising advancement in this field proved to be the coupling of CE with TOF mass spectrometry. As mentioned in section 4.2.3, the introduction of high resolution MS with TOF analysers highly increases analytical selectivity and, by simultaneous acquisition of the entire ion spectrum, allows for post-run retrieval of the signals of any ions of potential interest.

With regards to forensic toxicology, different analytical approaches based on CE-TOF MS for determination and quantitation of drugs of abuse for forensic purposes have been reported. For example, for the analysis of matrices simpler than biological fluids, such as seized drug samples, Lazar et al. described in 1998 the use of continuous infusion into an ESI-TOF MS through a CZE instrument [94]. The investigated molecules were tetracaine, procaine, methamphetamine, cocaine, amphetamine and heroin. A year later, the same group, proposed a peculiar CZE-ESI (micro spray)-TOF MS method for opium powder rough extracts, achieving a

LOD in the fmol range [95]. In addition, CE coupled to TOF MS was also applied for chiral analysis by using CDs as chiral selectors. Merola et al. used β-CD and HS-γ-CD for achieving the chiral separation of 12 cathinone analogues, belonging to the benzoylethanamines class and sold illegally as "bath salts", by means of PF-CZE-ESI-TOF MS [42]. The method provided chiral resolution and identification of individual analytes present in the mixture by determination of the exact mass by TOF MS. The method was tested using a small set of commercial "bath salts" and finally applied to seized drug sample. HS-γ-CDs were also the chiral selectors of choice for the simultaneous chiral separation of nine amphetamine-type stimulants by Reverse Polarity (RP) CZE with electrospray positive ionisation QTOF MS. This method was also applied to the analysis of trace precursors in illicit methamphetamine seizures [43].

On these grounds and on the basis of the experience of our research group in the hyphenation of CE with MS for toxicological analyses [96-98,17,99,100], the present work was focused on testing the hyphenation CE–TOF-MS with the final aim of comparing its efficiency *vs.* CE-UV, where peak broadening could represent a major issue in forensic toxicology validation of methods.

Also, the CE-MS coupling was tested in terms of susceptibility to ion-suppression, a phenomenon which plagues any use of MS with biological solutions.

8.2 MATERIALS AND METHODS

8.2.1 STANDARDS AND CHEMICALS

All used chemicals were of analytical grade. Isopropanol, ammonium formate and ammonia used for the preparation of CE buffers and for the ESI sheath liquids were purchased from Carlo Erba Reagents (Milan, Italy). NaCl and NaOH were also obtained from Carlo Erba Reagents. Formic acid was obtained from Sigma Aldrich (Steinheim, Germany). Milli-Q water, produced from Millipore Ultra-Pure Water system (Millipore, Bedford, MA, USA) was used for preparation of the buffers and all aqueous solutions. The running electrolyte solution was 25 mM ammonium formate, adjusted to pH 9.5 with ammonia; before use, the solution was filtered through 0.45 µm cellulose membranes and degassed under vacuum (water pump). Commercially available ready-to-use solutions for liquid–liquid extraction (ToxiVials A™) of basic compounds from biological samples were obtained from Interchim (Montluçon, France). Standards of 3,4-methylenedioxyamphethamine (MDA) and 3,4-methylenedioxymethamphethamine (MDMA) were provided by Salars (Como, Italy).

Stock solutions of the standards were prepared in methanol at individual concentrations of 2 mg/mL and stored at −20 °C until analysis. Working solutions were prepared in water and used daily.

8.2.2 CE–MS INSTRUMENTATION AND ANALYTICAL CONDITIONS

In the present study, an Agilent 7100 Capillary Electrophoresis System (Agilent Technologies, Santa Clara, CA, USA) was used. Untreated fused-silica capillaries of 75 µm i.d. ID, 100 cm total length (Agilent Technologies) were used, directly connected to the TOF MS at their cathodic end. The CE instrument was placed on a platform that was adjustable in height and position to avoid siphoning effects between the two ends of the capillary, which in the used CE–MS system were connected to two separated instruments.

The employed parameters of CE–MS coupling, previously reported in [98], were as follows. Separation voltage was applied at 15 kV in forward polarity. Temperature of the cooling liquid was kept at 20 °C. However, for practical problems due to the hindrance of the two instruments, which were interfaced, only the first 20 cm of the capillary were thermally regulated, whereas the remaining part was at room temperature. Hydrodynamic injection of the sample was carried out for 30 s at 100 mbar. Prior to each sample injection, the capillary was rinsed for 2 min at 1 bar with the running buffer. The terminal (cathodic) end of the capillary was connected to the orthogonal ESI source of a MicrOTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Before its first use, the capillary was sequentially rinsed with 1 M NaOH, Millipore water, and running buffer for 10 min each. All capillary rinses with NaOH were performed off-line at 1 bar with the ESI voltage switched off to avoid spray formation and the unnecessary entrance of NaOH in the MS. The on-line coupling was achieved with a commercial coaxial sheath liquid interface (Agilent Technologies, Santa Clara, CA, USA), which was positioned orthogonally to the MS ion source, the spray needle being grounded. Capillary voltage was −4 kV, source temperature 200 °C. Nitrogen was used both as drying and nebulising gas (drying gas flow rate: 5 L/min, nebuliser pressure: 0.6 bar). The mass spectrometer was operated in the positive ion scan mode from 50 to 800 m/z, with acquisition scan rate of 20 spectra per second. External calibration was obtained by infusing for 2 min at the beginning of each electrophoretic run a solution composed of 10 mM sodium hydroxide in isopropanol and 0.2 % formic acid (1:1, v/v), using seven calibration ions corresponding to the formulas $Na(NaCOOH)_x$, with x ranging from 2 to 9. The nominal resolution of the instrument was 10,000 (FWHM), accuracy 5 ppm. A coaxial sheath liquid consisting of a mixture of isopropanol/water (1:1, v/v) added with 0.5% formic acid was delivered at 4 µL/min by a syringe pump (Kd Scientific, Holliston, MA). Data processing was fully carried out using Data Analysis software (Version 3.2, Bruker Daltonics).

8.2.3 SAMPLE COLLECTION AND PREPARATION

Urine blanks were collected from four healthy volunteers that had never used either the target drugs nor any other illicit drugs. Drug-fortified urine samples were obtained by spiking blank urine with appropriate standard solutions. Blanks were analysed in an analogous way as spiked samples.

Sample extraction was performed according to the procedure provided with the commercial ToxiVials A™ tubes. Briefly, 3 mL of sample were introduced in the extraction tubes, which were shaken for 5 min and centrifuged at 2,500 rpm for 5 min. 1.5 mL of the organic phase (supernatant) were separated from the aqueous layer and transferred into a new glass tube, where they were evaporated at room temperature under air flow. The dried extract was subsequently reconstituted with 300 µL of distilled water for CE-MS analysis.

8.3 RESULTS AND DISCUSSION

The first objective of the present tests was to evaluate the CE-MS coupling in terms of efficiency of the separation in comparison to CE-UV. As it is well known, the miniaturised environment in which the CE separation takes place limits band broadening and consequently assures high efficiencies of the separations which, for molecules with low diffusion coefficients (e.g. DNA fragments), may reach millions of theoretical plates. Of course, with small molecules such as the analytes of interest in the present study the typical efficiency of CE is lower, but still comparable to gas chromatography. The miniaturisation of the separation compartment, however, makes crucial the detrimental effect of post-column added volumes. For this reason, the standard configuration of CE-UV requires an "in-column" detection, a detection scheme that reduces to zero the post-column band broadening. This configuration cannot be adopted in CE-MS where the detector is located after the separation capillaries, from which it is physically separated by the ionisation source. For this reason, in CE-MS a significant loss of efficiency is expected, which should be preliminarily studied and limited into a frame of acceptability for the purposes of the analysis.

For example, a study on the application of CE-UV to MDA and MDMA analysis [101] reported efficiencies around 150,000 plates. It is worth mentioning, however, that in such work, the sample injection was performed by using a form of sample stacking, which inherently makes the injection plug shorter and consequently increases the efficiency.

The tests performed in the present study with MDA and MDMA in aqueous solutions gave efficiencies of about 40,000 plates for both analytes, as an average of the two following formulas of calculation: $N = 5.54\ (t/w_{1/2})^2$ and $N = 16\ (t/w)^2$, where

t = migration time

w = width of the peak

$w_{1/2}$ = width of the peak at half the maximum height.

These figures, although lower than those reported for CE-UV, look perfectly suitable for application of CE-MS in the “real world”, taking into special consideration that the same efficiency was calculated with extracts of a biological matrix, such as urine.

In the evaluation of the potential usefulness of the application of CE-MS in forensic analysis, further consideration was devoted to the phenomenon of ion suppression, *i.e.* the strong disturbance of the ionisation of the analytes in the ion source effected by ions co-existing in the matrix. This phenomenon strongly affects LC-MS [102], particularly when non-standardised matrices are to be analysed and when deuterated internal standards are not available.

There are few reports in the recent literature discussing the occurrence of this phenomenon in CE-MS, where, for a series of reasons inherent in the technology, ion suppression seems less relevant than in LC-MS. In short, the reasons are:

1. the miniaturisation of the injected amount of sample brings less interfering compound into the ion source;
2. the miniaturisation of the flow rate brings less electrolytes into the ion source;
3. the field applied in the CE capillary drives the small ions with a suitable charge towards the injection side of the capillary and consequently they do not enter the ion source;
4. the sheath flow (if it is applied in the interface) dilutes the liquid coming from the separation capillary which becomes more suitable for the electrospray ionisation.

On such grounds, experiments were conducted to verify in practice the ion suppression occurring in our system.

Since the interference from the matrix in CE-MS may occur both at the injection step (defocusing of the peak) and at the ionisation source (ion suppression), it was decided to skip the formal study of ion suppression as proposed by Matuszewski et al. [86].

The comparison of the peak areas of the analyte in the absence and in the presence of the matrix was performed, instead. In order to do this, a suitable volume of a blank matrix was extracted as described in section 8.2.3 and reconstituted with distilled water containing the analyte of interest at a suitable concentration. The peak areas resulting from the analysis of this solution were compared with those from the extraction from saline solution, also reconstituted with distilled water containing the same concentration of the analyte.

MDA at a concentration of 500 ng/mL produced a peak area of 6.03×10^6 when injected from the reconstitution of saline solution extract, whereas the injection of an extract of blank urine also reconstituted with the same concentration of MDA produced a peak area of 7.29×10^6.

MDMA at a concentration of 500 ng/mL produced a peak area of 11.89×10^6 when injected from the reconstitution of saline solution extract, whereas the injection of an extract of blank urine also reconstituted with the same concentration of MDMA produced a peak area of 13.53×10^6.

On these grounds, the matrix effect can be calculated as 120 % and 114 % for MDA and MDMA respectively, thus creating an ion enhancement effect instead of the expected ion suppression. It is worth mentioning that this effect was observed on a urine extract concentrated five times, and therefore the original concentration mimicked in these experiments is 100 ng/mL. It is also worth mentioning that the observed ion enhancement can be easily controlled by the use of a suitable deuterated internal standard.

Finally yet importantly, it is to be pointed out that the recovery of the simple liquid-liquid extraction method from urine at the analyte concentration used in the experiment was 73 % and 83 % for MDA and MDMA respectively (Figure 25).

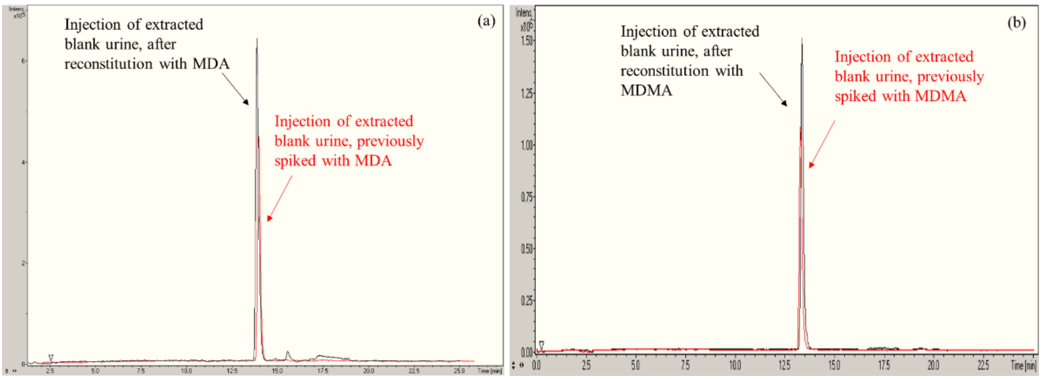


Figure 25. Overlay of peaks derived from pre- and post- extraction spiking with MDA (a) and MDMA (b) of blank urine samples. Extraction occurred according to procedure described in section 8.2.3.

8.4 PRELIMINARY CONCLUSIONS AND FUTURE DIRECTIONS

The experiments above described, although preliminarily, strongly support the perspectives of the coupling CE-MS in the analysis of compounds of forensic toxicological interest.

The coupling is quite simple, if the instrumentation can be fitted with a suitable interface, such as the tri-coaxial sheath liquid, which was used in the present work. The results show that this interface generates a stable spray and an efficient ionisation, despite the high dilution of the capillary effluent given by the sheath flow. In fact, the signal originated by the injection of tiny amounts of analytes (in the order of few pg) is remarkable, allowing sensitivities down to few ng/mL. These results were obtained by using pressure injection, and therefore improvements by a factor of at least 10-100 folds can be expected by using electrokinetic injection systems and particularly FASS. The presented approach showed also a good efficiency, despite the post-column effect given by the ESI. In fact, the theoretical plates count of our separations accounted for about 40,000 plates which are more than acceptable in the forensic toxicology field. Contrarily to our expectations, the biological matrix of urine produced ion enhancement instead of ion suppression. Even if the reason of this phenomenon has not been clarified, this can contribute to the improvement of the sensitivity of the technique for toxicological screening, where quantitative data are not required. However, in order to produce quantitative results, we strongly believe that the use of a deuterated internal standard could be helpful in the solution of the problem.

9. CONCLUDING REMARKS

Capillary electrophoresis, introduced in the late 1970s, experienced diversified fortunes in its application in chemical and biological analysis. On one side, it has become a major analytical technique in DNA sequencing and in protein analysis, but on the other side it has been substantially neglected, such as in pharmaceutical and chemical studies. In forensic toxicology and in the other analytical branches of forensic sciences, the penetration of CE has, up to now, been relatively scarce. Despite this poor appreciation in the forensic analytical practice, a sound body of literature [3] supports the potential usefulness of CE in many fields of forensics including inorganic and organic ion analysis, drug analysis, explosives and gunshot residue analysis, trace analysis, etc. The only field where CE has gained general consensus is DNA fingerprinting, as a consequence of its fortune in human genetics.

The purpose of the present work was to reconsider some of the crucial fields of application of CE where this analytical technique shows distinct advantages over the existing ones. In this frame, the work explained in detail in the present dissertation allowed the verification of the following rational hypotheses:

1. CE in its most simple mode of application (CZE with UV detection) is the most suitable and, probably, the easiest approach to face the problems posed by chiral analysis. This was proved through the development of a simple method for the chiral determination of ketamine and norketamine. The suitability of CZE in the presence of cyclodextrins as chiral selectors was verified also in a complex biological matrix such as human hair, including samples from real criminal cases. It worth noting that in-capillary UV detection proved sensitive enough for the analysis of samples containing ketamine concentration well below the ng/mg range, which is not far from the sensitivity of LC-MS. On the chiral selectivity side, CE offers the great advantage of using the chiral selector in solution instead of the chiral

columns required in LC, with a consequent reduction of costs and increase in flexibility. Similar considerations apply to the chiral analysis of baclofen, which however could not be applied to real samples because the lack of chromophores on the molecule hampered the application of UV detection.

2. CE was also studied in its possibility to be coupled with MS, which has become a sort of gold standard in forensic analyses. The preliminary tests conducted in the frame of the present work were fully satisfactory in terms of signal stability, analytical efficiency and easiness of handling. The tests were performed with two ring-substituted amphetamines such as MDA and MDMA which could easily and unequivocally be detected by high resolution MS in urine matrix, showing no detrimental matrix effects (instead of ion suppression, a 20 % ion enhancement).

In conclusion, the work described in the present dissertation consistently supports the usefulness of CE in forensic toxicological analysis. However, it must be taken into consideration that a proper use of this technique needs a sound background both in electrophoresis and in instrumental analysis, which rarely can be found in forensic analytics. In fact, electrophoresis is traditionally performed in the biological area, where instrumental analysis is rarely adopted or, when present, is used as a “fire-and-forget” weapon. On the other side, expertise in instrumental analysis is well present in analytical chemistry, where however electrophoresis is practically an unknown entity. This “cultural divide” is in our opinion the main obstacle to a better acceptance of CE in forensics, and this work is intended also to bridge this breach.

10. REFERENCES

1. Tagliaro F, Bortolotti F (2006) Recent advances in the applications of CE to forensic sciences (2001–2004). Electrophoresis 27:231-243

2. Tagliaro F, Bortolotti F (2008) Recent advances in the applications of CE to forensic sciences (2005–2007). Electrophoresis 29:260-268

3. Tagliaro F, Pascali J, Fanigliulo A, Bortolotti F (2010) Recent advances in the application of CE to forensic sciences: An update over years 2007–2009. Electrophoresis 31:251-259

4. Schmitt-Kopplin P, Englmann M (2005) Capillary electrophoresis – mass spectrometry: Survey on developments and applications 2003–2004. Electrophoresis 26:1209-1220

5. Evans CE, Stalcup AM (2003) Comprehensive strategy for chiral separations using sulfated cyclodextrins in capillary electrophoresis. Chirality 15:709-723

6. Sánchez-López E, Marina ML, Crego AL (2016) Improving the sensitivity in chiral capillary electrophoresis. Electrophoresis 37:19-34

7. Harris DC (2007) Quantitative Chemical Analysis. 7th edn.,

8. Moffat AC, Osselton MD, Widdop B, Watts J (2004) Clarke's Analysis of Drugs and Poisons in Pharmaceuticals, Body Fluids and Postmortem Material. third edn., Pharmaceutical Press, London, UK

9. Krylov SN, Starke DA, Arriaga EA, Zhang Z, Chan NWC, Palcic MM, Dovichi NJ (2000) Instrumentation for Chemical Cytometry. Analytical Chemistry 72:872-877

10. Kuffner CA, Marchi E, Morgado JM, Rubio CR (1996) Peer Reviewed: Capillary Electrophoresis and Daubert: Time for Admission. Analytical Chemistry 68 (7):241A-246A.

11. Hernández-Borges J, Borges-Miquel TM, Rodríguez-Delgado MÁ, Cifuentes A (2007) Sample treatments prior to capillary electrophoresis–mass spectrometry. Journal of Chromatography A 1153 (1–2):214-226.

12. Al-Othman ZA, Dawod M, Kim JY, Chung DS (2012) Single-drop microextraction as a powerful pretreatment tool for capillary electrophoresis: A review. Analytica Chimica Acta 739:14-24.

13. He H, Liu S, Meng Z, Hu S (2014) Dispersive liquid–liquid microextraction for the determination of phenols by acetonitrile stacking coupled with sweeping-micellar electrokinetic chromatography with large-volume injection. Journal of Chromatography A 1361:291-298.

14. Zhou X, He M, Chen B, Hu B (2015) Hollow fiber based liquid-liquid-liquid microextraction combined with sweeping micellar electrokinetic chromatography for the sensitive determination of second-generation antidepressants in human fluids. Analyst 140 (5):1662-1671.

15. De Rossi A, Desiderio C (2006) High sensitivity analysis of oxprenolol in urine by capillary electrophoresis with C18 packed on-line preconcentrator. Journal of Chromatography B 839 (1–2):6-11.

16. Hernández-Borges J, Rodríguez-Delgado MÁ, García-Montelongo FJ, Cifuentes A (2004) Highly sensitive analysis of multiple pesticides in foods combining solid-phase microextraction, capillary electrophoresis-mass spectrometry, and chemometrics. Electrophoresis 25 (13):2065-2076.

17. Gottardo R, Polettini A, Sorio D, Pascali J, Bortolotti F, Liotta E, Tagliaro F (2008) Capillary zone electrophoresis (CZE) coupled to time-of-flight mass spectrometry (TOF-MS) applied to the analysis of illicit and controlled drugs in blood. Electrophoresis 29 (19):4078-4087.

18. Wey AB, Thormann W (2001) Head-column field-amplified sample stacking in presence of siphoning Application to capillary electrophoresis–electrospray ionization mass spectrometry of opioids in urine. Journal of Chromatography A 924:507-518

19. Isoo K, Otsuka K, Terabe S (2001) Application of sweeping to micellar electrokinetic chromatography-atmospheric pressure chemical ionization-mass spectrometric analysis of environmental pollutants. Electrophoresis 16:3426-3432

20. Scriba GKE (2007) Nonaqueous capillary electrophoresis–mass spectrometry. Journal of Chromatography A 1159:28-41

21. Jussila M, Sinervo K, Porras SP, Riekkola ML (2000) Modified liquid junction interface for nonaqueous capillary electrophoresis-mass spectrometry. Electrophoresis 21:3311-3317

22. Varjo SJO, Jussila M, Palonen S, Riekkola ML (2002) Interface for coupling nonaqueous wide-bore capillary electrophoresis with mass spectrometry. Electrophoresis 23 (3):437-441.

23. Huhn C, Ramautar R, Wuhrer M, Somsen GW (2010) Relevance and use of capillary coatings in capillary electrophoresis–mass spectrometry. Analytical Bioanalytical Chemistry 396:297-314

24. Vanhoenacker G, De l'Escaille F, De Keukeleire D, Sandra P (2004) Analysis of Benzodiazepines in Dynamically Coated Capillaries by CE-DAD, CE-MS and CE-MS^2. Journal of Pharmaceutical and Biomedical Analysis 34:595-606

25. Kohler I, Schappler J, Rudaz S (2013) Highly sensitive capillary electrophoresis-mass spectrometry for rapid screening and accurate quantitation of drugs of abuse in urine. Analytica Chimica Acta 780:101-109

26. Peterson ZD, Bowerbank CR, Collins DC, Graves SW, Lee ML (2003) Advantages and limitations of coupling isotachophoresis and comprehensive isotachophoresis–capillary electrophoresis to time-of-flight mass spectrometry. Journal of Chromatography A 992:169–179

27. Mazereeuw M, Spikmans V, Tjaden UR, Van der Greef J (2000) On-line isotachophoretic sample focusing for loadability enhancement in capillary electrochromatography–mass spectrometry. Journal of Chromatography A 879:219-233

28. Wernly P, Thormann W (1991) Analysis of Illicit Drugs in Human Urine by Micellar Electrokinetic Capillary Chromatography with On-Column Fast Scanning Polychrome Absorption Detection. Analytical Chemistry 63:2878-2882

29. Somsen GW, Mol R, De Jong GJ (2003) On-line micellar electrokinetic chromatography–mass spectrometry: feasibility of direct introduction of non-volatile buffer and surfactant into the electrospray interface. Journal of Chromatography A 1000:953-961

30. Stubberud K, Callmer K, Westerlund D (2003) Partial filling-micellar electrokinetic chromatography optimization studies of ibuprofen, codeine and degradation products, and coupling to mass spectrometry: Part II. Electrophoresis 24:1008-1016

31. Von-Brocke A, Nicholson G, Bayer E (2001) Recent advances in CE-ESI-MS. Electrophoresis 22:1251–1266

32. Taylor MR, Teale P (1997) Gradient capillary electrochromatography of drug mixtures with UV and electrospray ionisation mass spectrometric detection. Journal of Chromatography A 768:89-95

33. Tagliaro F (2011) Capillary Electrophoresis. In: Press P (ed) Clarke's Analysis of Drugs and Poisons vol 1. 4th edn., pp 758-772

34. Sung W-C, Makamba H, Chen S-H (2005) Chip-based microfluidic devices coupled with electrospray ionization-mass spectrometry. Electrophoresis 26:1783-1791

35. Thorslund S, Lindberg P, Andrén PE, Nikolajeff F, Bergquist J (2005) Electrokinetic-driven microfluidic system in poly(dimethylsiloxane) for mass spectrometry detection integrating sample injection, capillary electrophoresis, and electrospray emitter on-chip. Electrophoresis 26:4674-4683

36. Nordman N, Sikanen T, Moilanen M-E, Aura S, Kotiaho T, Franssila S, Kostiainen R (2011) Rapid and sensitive drug metabolism studies by SU-8 microchip capillary electrophoresis-electrospray ionization mass spectrometry. Journal of Chromatography A 1218:739-745

37. Jáč P, Scriba GKE (2013) Recent advances in electrodriven enantioseparations. Journal of Separation Science 36:52-74

38. Fanali S (2000) Enantioselective determination by capillary electrophoresis with cyclodextrins as chiral selectors. Journal of Chromatography A 875:89-122

39. Ingelse BA (1997) Chiral separations using capillary electrophoresis. doi:10.6100/IR492451

40. Kwan HY, Thormann W (2011) Enantioselective capillary electrophoresis for the assessment of CYP3A4-mediated ketamine demethylation and inhibition in vitro. Electrophoresis 32:2738-2745

41. Vaccher MP, Lipka E, Bonte JP, Foulon C, Goossens JF, Vaccher C (2005) Enantiomeric analysis of baclofen analogs by capillary zone electrophoresis, using highly sulfated cyclodextrins: Inclusion ionization constant pKa determination. Electrophoresis 26:2974-2983

42. Merola G, Fu H, Tagliaro F, Macchia T, McCord BR (2014) Chiral separation of 12 cathinone analogs by cyclodextrin-assisted capillary electrophoresis with UV and mass spectrometry detection. Electrophoresis 35:3231-3241

43. Iwata YT, Kanamori T, Ohmae Y, Tsujikawa K, Inoue H, Kishi T (2003) Chiral analysis of amphetamine-type stimulants using reversed-polarity capillary electrophoresis/ positive ion electrospray ionization tandem mass spectrometry. Electrophoresis 24:1770-1776

44. Cherkaoui S, Veuthey JL (2002) Use of negatively charged cyclodextrins for the simultaneous enantioseparation of selected anesthetic drugs by capillary electrophoresis-mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 27:615-626

45. Rudaz S, Calleri E, Geiser L, Cherkaoui S, Prat J, Veuthey JL (2003) Infinite enantiomeric resolution of basic compounds using highly sulfated cyclodextrin as chiral selector in capillary electrophoresis. Electrophoresis 24:2633-2641

46. Rudaz S, Geiser L, Souverain S, Prat J, Veuthey JL (2005) Rapid stereoselective separations of amphetamine derivatives with highly sulfated g-cyclodextrin. Electrophoresis 26:3910-3920

47. Gross JH, Davoli E, Medana C (2016) Spettrometria di massa. 1 edn.,

48. Grim NC, Sarquis JL (1995) Mass Spectrometry Analogy on the Overhead Projector. Journal of Chemical Education 72 (10):930. doi:10.1021/ed072p930

49. Brunnée C (1987) The ideal mass analyzer: Fact or fiction? International Journal of Mass Spectrometry and Ion Processes 76:125-237

50. Walker JA, Krueger ST, Lurie IS, Marché HL, Newby N (1995) Analysis of heroin drug seizures by Micellar Electrokinetic Capillary Chromatography (MECC). Journal of Forensic Sciences 40:6-9

51. Pai A, Heining M (2007) Ketamine. Continuing Education in Anaesthesia, Critical Care & Pain 7 (2):59-63

52. Theurillat R, Knobloch M, Schmitz A, Lassahn PG, Mevissen M, Thormann W (2007) Enantioselective analysis of ketamine and its metabolites in equine plasma and urine by CE with multiple isomer sulfated β-CD. Electrophoresis 28 (15):2748-2757.

53. Theurillat R, Knobloch M, Levionnois O, Larenza P, Mevissen M, Thormann W (2005) Characterization of the stereoselective biotransformation of ketamine to norketamine via determination of their enantiomers in equine plasma by capillary electrophoresis. Electrophoresis 26:3942-3951

54. Schmitz A, Thormann W, Moessner L, Theurillat R, Helmja K, Mevissen M (2010) Enantioselective CE analysis of hepatic ketamine metabolism in different species in vitro. Electrophoresis 31:1506-1516

55. Baselt RC, Cravey RH (1995) Disposition of Toxic Drugs and Chmicals in Man. fourth edn., Chemical Toxicology Institute, Foster City, CA

56. Craven R (2007) Ketamine. Anaesthesia 62:48-53

57. Sinner B, Graf BM (2008) Ketamine. In: Schüttler J, Schwilden H (eds) Modern Anesthetics, vol 182. Handbook of Experimental Pharmacology. Springer Berlin Heidelberg, pp 313-333.

58. Aroni F, Iacovidou N, Dontas I, Pourzitaki C, Xanthos T (2009) Pharmacological aspects and potential new clinical applications of ketamine: reevaluation of an old drug. J Clin Pharmacol 49 (8):957-964

59. Machado-Vieira R, Salvadore G, Granados ND, Zarate CA (2009) Ketamine and the next generation of antidepressants with a rapid onset of action. Pharmacol Therapeut 123:143-150

60. Cheng PS, Lee CH, Liu C, Chien CS (2008) Simultaneous determination of ketamine, tramadol, methadone, and their metabolites in urine by gas chromatography-mass spectrometry. J Anal Toxicol 32:253-259

61. Kim EM, Lee JS, Choi SK, Lim MA, Chung HS (2008) Analysis of ketamine and norketamine in urine by automatic solid-phase extraction (SPE) and positive ion chemical ionization–gas chromatography–mass spectrometry (PCI–GC–MS). Forensic Sci Int 174:197-202

62. Adamowicz P, Kala M (2005) Urinary excretion rates of ketamine and norketamine following therapeutic ketamine administration: method and detection window considerations. J Anal Toxicol 28:376-382

63. Cheng PS, Fu CY, Lee CH, Liu C, Chien CS (2007) GC–MS quantification of ketamine, norketamine, and dehydronorketamine in urine specimens and comparative study using ELISA as the preliminary test methodology. J Chromatogr B 852:443-449

64. Lin HR, Lua AC (2006) Simultaneous determination of amphetamines and ketamines in urine by gas chromatography/mass spectrometry. Rapid Commun Mass Sp 20:1724-1730

65. Wang KC, Shihb TS, Chenga SG (2005) Use of SPE and LC/TIS/MS/MS for rapid detection and quantitation of ketamine and its metabolite, norketamine, in urine. Forensic Sci Int 147:81-88

66. Cheng JYK, Mok VKK (2004) Rapid determination of ketamine in urine by liquid chromatography–tandem mass spectrometry for a high throughput laboratory. Forensic Sci Int 142:9-15

67. Fernandez MdMR, Laloup M, Wood M, De Boeck G, Lopez-Rivadulla M, Wallemacq P, Samyn N (2007) Liquid Chromatography-Tandem Mass Spectrometry Method for the Simultaneous Analysis of Multiple Hallucinogens, Chlorpheniramine, Ketamine, Ritalinic Acid, and Metabolites, in Urine. J Anal Toxicol 31:497-504

68. Chen CY, Lee MR, Cheng FC, Wu GJ (2007) Determination of ketamine and metabolites in urine by liquid chromatography–mass spectrometry. Talanta 72:1217-1222

69. Parkin MC, Turfus SC, Smith NW, Halket JM, Braithwaite RA, Elliott SP, Osselton MD, Cowan DA, Kicman AT (2008) Detection of ketamine and its metabolites in urine by ultra high pressure liquid chromatography–tandem mass spectrometry. J Chromatogr B 876:137-142

70. Chen LC, Hashimoto Y, Furuya H, Takekawa K, Kubota T, Hiraoka K (2009) Rapid detection of drugs in biofluids using atmospheric pressure chemi/chemical ionization mass spectrometry. Rapid Commun Mass Sp 23:333-339

71. Lin YH, Li JH, Ko WK, Wu SM (2006) Direct and sensitive analysis of methamphetamine, ketamine, morphine and codeine in human urine by cation-selective exhaustive injection and sweeping micellar electrokinetic chromatography J Chromatogr A 1130:281-286

72. Jen HP, Tsai YC, Su HL, Hsieh YZ (2006) On-line preconcentration and determination of ketamine and norketamine by micellar electrokinetic chromatography: Complementary method to gas chromatography/mass spectrometry. J Chromatogr A 1111:159-165

73. Aboul-Enein HY, Hefnawy MM (2005) Liquid chromatographic high-throughput analysis of ketamine and its metabolites in human plasma using a monolithic silica column and solid phase extraction. Talanta 65:67-73

74. Sergi M, Bafile E, Compagnone D, Curini R, D' Ascenzo G, Romolo FS (2009) Multiclass analysis of illicit drugs in plasma and oral fluids by LC-MS/MS. Anal Bioanal Chem 393:709-718

75. Favretto D, Vogliardi S, Stocchero G, Nalesso A, Tucci M, Terranova C, Ferrara SD (2013) Determination of ketamine and norketamine in hair by micropulverized extraction and liquid-chromatography-high resolution mass spectrometry. Forensic Sci Int 226:88-93

76. Sporkert F, Pragst F (2000) Use of headspace solid-phase microextraction (HS-SPME) in hair analysis for organic compounds. Forensic Sci Int 107:129-148

77. Gentili S, Cornetta M, Macchia T (2004) Rapid screening procedure based on headspace solid-phase microextraction and gas chromatography–mass spectrometry for the detection of many recreational drugs in hair. J Chromatogr B 801:289-296

78. Leong HS, Tan NL, Lui CP, Lee TK (2005) Evaluation of Ketamine Abuse Using Hair Analysis: Concentration Trends in a Singapore Population. J Anal Toxicol 29:314-318

79. Xiang P, Shen M, Zhuo X (2006) Hair analysis for ketamine and its metabolites. Forensic Sci Int 162:131-134

80. Kim JY, In MK, Kim JH (2006) Determination of ketamine and norketamine in hair by gas chromatography/mass spectrometry using two-step derivatization. Rapid Commun Mass Sp 20 3159–3162

81. Tabernero MJ, Felli ML, Bermejo AM, Chiarotti M (2009) Determination of ketamine and amphetamines in hair by LC/MS/MS. Anal Bioanal Chem 395:2547-2557

82. Harun N, Anderson RA, Cormack PA (2010) Analysis of ketamine and norketamine in hair samples using molecularly imprinted solid-phase extraction (MISPE) and liquid chromatography–tandem mass spectrometry (LC-MS/MS). Anal Bioanal Chem 396:2449-2459

83. Lin YH, Lee MR, Lee RJ, Ko WK, Wu SM (2007) Hair analysis for methamphetamine, ketamine, morphine and codeine by cation-selective exhaustive injection and sweeping micellar electrokinetic chromatography. J Chromatogr A 1145 (1–2):234-240.

84. Peters FT, Drummer OH, Musshoff F (2007) Validation of new methods. Forensic Sci Int 165:216-224

85. Musshoff F, Madea B (2007) New trends in hair analysis and scientific demands on validation and technical notes. Forensic Sci Int 165:204-215

86. Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003) Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC−MS/MS. Anal Chem 75 (13):3019-3030.

87. Vaccher MP, Lipka E, Bonte JP, Vaccher C (2004) Chiral capillary electrophoretic resolution of baclofen, gabaergic ligand, using highly sulfated cyclodextrins. Electrophoresis 25:1111-1120

88. Bowery NG (1982) Baclofen: 10 years on. Trends in Pharmacolgical Sciences 3:400-403

89. Frydenvang K, Hansen JJ, Krogsgaard-Larsen P, Mitrovic A, Tran H, Drew CA, Johnston GAR (1994) GABAB antagonists: Resolution, absolute stereochemistry, and pharmacology of (R)- and (S)-phaclofen. Chirality 6:583-589

90. Chiang MT, Chang SY, Whang CW (2001) Chiral analysis of baclofen by α-cyclodextrin-modified capillary electrophoresis and laser-induced fluorescence detection. Electrophoresis 22:123-127

91. Batra S, Bhushan R (2017) Enantioresolution of (RS)-baclofen by liquid chromatography: A review. Biomedical Chromatography 31:1-15

92. Ali I, Aboul-Enein HY (2003) Optimization of the chiral resolution of baclofen by capillary electrophoresis using β-cyclodextrin as the chiral selector. Electrophoresis 24:2064-2069

93. Olivares JA, Nguyen NT, Yonker CR, Smith RD (1987) On-Line Mass Spectrometric Detection for Capillary Zone Electrophoresis. Analytical Chemistry 59:1230-1232

94. Lazar IM, Lee Ed, Rockwood AL, Lee ML (1998) General considerations for optimizing a capillary electrophoresis– electrospray ionization time-of-flight mass spectrometry system. Journal of Chromatography A 829:279-288

95. Lazar IM, Naisbitt G, Lee ML (1999) Capillary electrophoresis time-of-flight mass spectrometry of an opium powder. Chromatographia 50:188-194

96. Gottardo R, Bortolotti F, Trettene M, De Paoli G, Tagliaro F (2004) Rapid and direct analysis of g-hydroxybutyric acid in urine by capillary electrophoresis–electrospray ionization ion-trap mass spectrometry. Journal of Chromatography A 1051:207-211

97. Gottardo R, Bortolotti F, De Paoli G, Pascali J, Mikˇs´ık I, Tagliaro F (2007) Hair analysis for illicit drugs by using capillary zone electrophoresis-electrospray ionization-ion trap mass spectrometry. Journal of Chromatography A 1159:185-189

98. Gottardo R, Fanigliulo A, Bortolotti F, De Paoli G, Pascali J, Tagliaro F (2007) Broad-spectrum toxicological analysis of hair based on capillary zone electrophoresis–time-of-flight mass spectrometry. Journal of Chromatography A 1159:190-197

99. Gottardo R, Fanigliulo A, Sorio D, Liotta E, Bortolotti F, Tagliaro F (2012) Monitoring compliance to therapy during addiction treatments by means of hair analysis for drugs and drug metabolites using CZE-TOF-MS. Forensic Science International 216:101-107

100. Gottardo R, Mikšík I, Aturki Z, Sorio D, Seri C, Fanali S, Tagliaro F (2012) Analysis of drugs of forensic interest with capillary zone electrophoresis/time-of-flight mass spectrometry based on the use of non-volatile buffers. Electrophoresis 33:599-606

101. Tagliaro F, Manetto G, Crivellente F, Scarcella D, Marigo M (1998) Hair analysis for abused drugs by capillary zone electrophoresis with field-amplified sample stacking. Forensic Science International 92:201-211

102. Lee S, Kim J, In S, Choi H, Oh SM, Jang CG, Chung KH (2012) Development of a simultaneous analytical method for selected anorectics, methamphetamine, MDMA, and their metabolites in hair using LC-MS/MS to prove anorectics abuse. Analytical Bioanalytical Chemistry 403:1385-1394

APPENDIX 1

Although the main doctoral research projects have been described in detail in the present thesis, a minor study was furthermore carried out. Being a research study based on a different topic (though belonging to forensic toxicology), it will be briefly reported in the present appendix.

Introduction

On the grounds of sound literature, carbohydrate deficient transferrin (CDT) is today widely accepted as the most specific biomarker of chronic abuse of alcohol. CDT is the collective name of a group of minor glycoforms of transferrin (Tf) including traditionally asialo-, monosialo-, and disialo-Tf. Among these isoforms, asialo-Tf is characterised by extremely low concentrations, which make its detectability in biological samples strongly dependent on the efficiency of the separation method and on the sensitivity of detection.

IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) recently recommended the exclusion of asialo-Tf from the computation of CDT to help the standardisation of the analytical procedures [1]. However, in our opinion, asialo-Tf could provide precious additional information to confirm, particularly in a forensic context, the diagnostic meaning of an elevated CDT result, based merely on disialo-Tf measurement. On this basis, in view of a re-evaluation of the potential of asialo-Tf as an additional biomarker of alcohol abuse, the purpose this project was to compare the two most popular separative methods for CDT determination, such as Capillary Zone Electrophoresis (CZE) and HPLC, in the ability to detect asialo-Tf in real serum samples.

[1] Schellenberg F, Wielders J, Raymond A, Bianchi V, Deenmamode J, Weykamp C, Withfield J, Jeppsson J-O, Helander A, *IFCC approved HPLC reference measurement procedure for the alcohol consumption biomarker carbohydrate-deficient trasnferrin (CDT): Its validation and use,* Clin Chimica Acta, doi: 10.1016/j.cca.2016.12.022

Materials and methods

All serum samples (n=103) were analysed for CDT investigation *via* both CZE and HPLC methods. Analytical conditions are summarised below:

Analysis of CDT by CZE

- Capillary electropherograph: P/ACE MDQ, equipped with a UV detector
- Running buffer: 120 mM H_3BO_3, pH 8.2 (reached by 6 M NaOH) and 6 mM DAB (1,4-diaminobutane)
- Hydrodynamic injection: 0.5 psi x 25 seconds
- Separation voltage and polarity: 30 kV, normal polarity
- Cartridge temperature: 25°C
- Capillary features: uncoated fused silica 30 µm i. d. x 60 cm T. L.
- Detection: UV absorbance at 200 nm
- Sample preparation prior to injection: the serum samples were saturated with a commercial ready-to-use ferric solution.

Analysis of CDT by HPLC

- HPLC system: Shimadzu HPLC, equipped with a UV-visible detector.
- Chromatography by salt gradient elution
- Column: anion-exchange column [65 x 4.6 mm (i.d.)] provided by the “ClinRep® CDT in Serum” commercial kit
- Starting phase A: 10 mM Bis-Tris, pH 6.2
- Phase B: 10 mM Bis-Tris and 500 mM NaCl, pH 6.2

- Phase C: 2 M NaCl, used for regeneration
- Sample injection: 100 µL
- Flow rate: 1 mL/min
- Detection: absorbance at 460 nm
- Sample preparation prior to injection: the serum samples were treated for lipoprotein precipitation with $CaCl_2$ and saturated with a commercial ready-to-use ferric solution.

Peak identification was accomplished by relative migration time analysis.

Finally, data were analysed by using non-parametric and parametric statistics to study the internal correlation of the concentrations of the different components of CDT (asialo- and disialo-Tf) and to compare the ability of CZE and HPLC to detect asialo- and disialo-Tf.

Results

A total of 103 serum specimens were analysed in the present study: 49 were from individuals undergoing compulsory control for certification to obtain a new driving license after theirs had been confiscated for driving under the influence of alcohol. The remaining 54 samples derived from individuals who were tested for alcohol abuse after being involved in a road accident. All serum samples were stored frozen at - 20 °C prior to analysis, which occurred in parallel with CZE and HPLC on the same day.

For both CZE and HPLC, the sum of areas for all transferrin isoforms was considered as 100% and areas for individual isoforms were reported as a percentage of the total amount of transferrin. While CDT isoform quantification (%CDT) was based on calculation of the percentage ratio of the (asialo-Tf + disialo-Tf) peak area to the sum of the peak areas for all iso-transferrin forms from asialo-Tf to pentasialo-Tf.

The cut-off between alcohol non-abusers and alcohol abusers was established at the 97.5th percentile from a group of 100 healthy blood donors, including social drinkers, who had no laboratory evidence of alcohol abuse or any alcohol-related disease and had a declared daily alcohol intake $\leq$ 50 g [2]. The resulting %CDT cut-off values were 1.8 % for CZE, and 1.9 % for HPLC.

Both CZE and HPLC provided excellent results in terms of limit of quantification (lowest CDT concentration measurable with a relative SD $\leq$ 20 %), the limits of quantification were 0.24 % CDT for CZE and 0.10 % CDT for HPLC.

Because its detection wavelength (460 nm) corresponded to the maximum absorbance of the ferric iron, HPLC was more selective than CZE, which used ultraviolet absorbance at 200 nm. On the other hand, the CZE method was more rapid, requiring simpler sample preparation, although overall analysis times (including separation, cleaning, and reconditioning of the system) were comparable between the two methodologies.

Specifically, concerning the analytical sensitivity in detecting the disialo-Tf, the correlation between CZE and HPLC is expressed by the linear regression $y = 1.177 x - 0.158$ ($r^2 = 0.970$) indicating a highly significant correlation between the two methods.

On the other hand, the detection ability for asialo-Tf was extremely higher for CZE than for HPLC. Both in CZE and HPLC, 32 of the 103 samples analysed resulted positive for CDT with values higher than 1.8% and 1.9% respectively. However, 28 out of 32 showed the presence of asialo-Tf when analysed in CZE and only 13 of the 32 when tested in HPLC. The range detected varied between 0.10 – 3.78 % in CZE and 0.12 – 1.61 % in HPLC.

Because of such scarce number of asialo-Tf detected in HPLC (n=13), the linear regression determining the correlation between asialo- and disialo-Tf was not measured. On the contrary, such correlation measured for CZE results (n=28) provided the following results: $y = 0.311 x - 0.330$ ($r^2 = 0.524$).

Finally the separation efficiency measured in plates number/column and expressed by the formula $N = 5.54 \cdot (t / w_{1/2})^2$ proved CZE to be extremely more unfailing than HPLC with 51,000 plates/column vs. 4,200 plates/column.

[2] Bortolotti F, De Paoli G, Pascali J P, Trevisan M T, Floreani M, Tagliaro F, *Analysis of Carbohydrate-Deficient Transferrin: Comparative Evaluation of Turbidimetric Immunoassay, Capillary Zone Electrophoresis, and HPLC*, Clin Chem 2005; 51:2368-71.

Discussion and future directions

Alcohol abuse, definable as "a pattern of alcohol use leading to clinically significant impairment", is often the cause of disregard for others, poor self-control and failure to fulfil major commitments related to family, school or work. Such issues are the reasons why it is mandatory to verify a chronic alcohol abuse in various situations of different forensic contexts.

Such controls are daily performed in our lab by measuring the value of a heavy drinking biomarker, CDT, which refers to the presence in serum of minor transferrin isoforms and, in particular disialo-Tf. After a period of heavy drinking (>60 g ethanol per day, for several weeks), the result is a decreased hepatic glycosylation of Tf, leading to a decrease in the tetrasialo component and a proportional increase in the disialo form.

Asialo-Tf could, however, provide precious additional information to confirm, particularly in a forensic context, the diagnostic meaning of an elevated CDT result, based merely on disialo-Tf measurement. On this basis, asialo-Tf could also become an additional, possibly more specific, biomarker of alcohol abuse.

A number of different methods exist for measuring CDT in serum, with CZE and HPLC being the most commonly widespread. Notwithstanding a comparable performance of CZE and HPLC in the determination of disialo-Tf, the ability of CZE to detect asialo-Tf is clearly superior. This can be ascribed to a neatly better efficiency of the electrophoretic separation of Tf glycoforms, providing sharper and more detectable peaks (CZE 51,000 plates/column vs. HPLC 4,200 plates/column). In addition, the asialo-Tf peak is located in a very flat region of the electropherogram, far from other potentially interfering peaks. The present results strongly support the use of CZE in further studies and at exploring the potential of asialo-Tf as an additional, important biomarker of alcohol abuse, alcohol-related pathologies and traffic and industrial accidents.

The present study has the aim to compare, in a detailed manner, the differences between two methodologies validated for the determination of chronic alcohol abuse by means of a valuable biomarker such as CDT. Therefore, extending such comparison to a wider number of samples is the main future direction to undertake. Moreover, samples derived from different pools other than the two used up to date would also add higher variability to the comparison.

In view of a re-evaluation of the potential of asialo-Tf as an additional biomarker of alcohol abuse and of related pathologies, an additional future task will be to replicate the analysis of sample by using a different CE instrumentation, possibly showing a higher sensitivity in the asialo-Tf detection.

With regards to the productivity of the method, future studies could also be oriented towards a sort of automation of the process, possibly relating to the sample preparation section.

Finally, concerning the detection, hence the sensitivity of the method, this could possibly be improved by means of a more sensitive detection. For example, fluorescence detection could be applied to try to pursue such goal.

ABBREVIATIONS

ACN Acetonitrile

APCI Atmospheric-Pressure Chemical Ionisation

BGE Background Electrolyte

CDs Cyclodextrins

CDT Carbohydrate Deficient Transferrin

CE Capillary Electrophoresis

CEC Capillary Electro-Chromatography

CE-MS Capillary Electrophoresis - Mass Spectrometry

CGE Capillary Gel Electrophoresis

CI Chemical Ionisation

CITP Capillary Isotachophoresis

CNS Central Nervous System

CZE Capillary Zone Electrophoresis

EI Electron Ionisation

EKI Electrokinetic Injection

EOF Electroosmotic Flow

ESI Electrospray Ionisation

FASS Field-Amplified Sample Stacking

FWHM Full Width at Half Maximum

GABA γ-amino-butyric acid

HP Hydroxypropyl

HS Highly Sulphated

IT Ion Trap

LVI Large Volume Injection

MALDI Matrix-Assisted Laser Desorption Ionisation

MDA 3,4-methylenedioxyamphethamine

MDMA 3,4-methylenedioxymethamphethamine

MEKC Micellar Electrokinetic Chromatography

MS Mass Spectrometry

MT Migration Time

m/z Mass-to-charge ratio

NACE Non-Aqueous Capillary Electrophoresis

NMDA *N*-methyl-D-aspartate

NPSs New Psychoactive Substances

PEO Poly-(ethylene glycol)

PF Partial Filling

Q Quadrupole

RP Reverse Polarity

S Sulphated

SIM Single Ion Monitoring

SPE Solid Phase Extraction

Tf Transferrin

TOF Time-Of-Fligh

SCIENTIFIC PUBLICATIONS

N. Porpiglia, G. Musile, F. Bortolotti, E. F. De Palo, F. Tagliaro - **Chiral separation and determination of ketamine and norketamine in hair by capillary electrophoresis**, *Forensic Science International. 2016 Sep; 266: 304-10*

N. Porpiglia, E. Giacomazzi, R. Gottardo, F. Tagliaro - **CE-MS in Forensic Sciences with Focus on Forensic Toxicology**, *chapter of the book titled "Capillary Electrophoresis-Mass Spectrometry (CE-MS) – Principles and Applications" edited by Gerhardus de Jong – July 2016*

ORAL AND POSTER COMMUNICATIONS AT NATIONAL AND INTERNATIONAL SYMPOSIA

D. Sorio, A. Bertaso, N. Porpiglia, C. Tondi, F.Bortolotti – **Strategie analitiche alternative per lo studio dell'abuso alcolico cronico nel cadavere mediante Carbohydrate Deficient Transferrin (CDT) -** *Book of Abstracts, Giornata Giovani GTFI (Gruppo Tossicologi Forensi Italiani) 2016, Pavia Oct 28th 2016*

A. Bertaso, D. Sorio, N. Porpiglia, E. F. De Palo – **Finger-prick dried blood spot and capillary electrophoresis: A challenge for alcohol abusers screening analysis** – *Book of Abstracts, International Symposium on Electro- and Liquid Phase-Separation Techniques (ITP) 2015, Helsinki August 30th- September 3rd 2015*

N. Porpiglia, G. Musile, F. Bortolotti, E. F. De Palo, F. Tagliaro – **Chiral determination of ketamine and norketamine by capillary electrophoresis in hair specimens, as a tool to investigate histories of abuse –** *Book of Abstracts, 26th International Symposium on Pharmaceutical and Biomedical Analysis (PBA 2015), Tiblisi July 5th-8th* – Poster + Oral presentation

A. Bertaso, D. Sorio, N. Porpiglia, E. Giacomazzi, E. F. De Palo – **Development and preliminary use of CDT analysis on Dried Blood Spot (DBS) in forensic and administrative contexts –** *Book of Abstracts, Euromedlab 2015, Paris June 21st-25th 2015*

N. Porpiglia, R. Gottardo , F. Bortolotti, F. Tagliaro – **Analisi chirale della ketamina e del suo principale metabolita norketamina in matrici pilifere mediante elettroforesi capillare –** *Book of Abstracts, Giornata Giovani GTFI (Gruppo Tossicologi Forensi Italiani) 2015, Mestre April 28th 2015 – Oral presentation*

N. Porpiglia, G. Musile, R. Gottardo, F. Tagliaro, F. Bortolotti – **Rapid and simple chiral analysis of ketamine and norketamine in hair by capillary zone electrophoresis –** *Book of Abstracts, The International Association of Forensic Toxicologsts (TIAFT) 2014, Buenos Aires November 9th–13th 2014*

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