Review

Modern Instrumental Methods in Forensic Toxicology*

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Abstract

This article reviews modern analytical instrumentation in forensic toxicology for identification and quantification of drugs and toxins in biological fluids and tissues. A brief description of the theory and inherent strengths and limitations of each methodology is included. The focus is on new technologies that address current analytical limitations. A goal of this review is to encourage innovations to improve our technological capabilities and to encourage use of these analytical techniques in forensic toxicology practice.

Introduction

Instrumental methods are the cornerstone of modern forensic toxicology analyses. This review presents the theory and most recent applications of current technology for analysis of toxicants in biological fluids and tissues. The descriptions of methodological theory are necessarily brief, and readers who wish additional detail are referred to a more comprehensive text (1). We discuss methodological limitations and innovations that address these limitations. To fully understand instrument performance, one must be familiar with common characteristics of analytical assays. These include signal-to-noise ratio (S/N), limit of detection (LOD), lower limit of quantification (LOQ), upper limit of quantification (ULOQ), accuracy, precision, interference, and robustness. Because of the importance of these parameters for characterizing assays, they are integral components of method validation (2).

Most analytical instruments convert some property of an analyte into an electronic or photometric signal. Many factors, such as random electronic or spurious photon transmissions, fluctuating concentrations of inherent substances at the detector, and others, contribute unwanted signal that may interfere with measured target response. These aberrant signals are labeled noise. The best technologies improve analyte signal or reduce noise, or both, to increase S/N. High S/N results in lower LOD and LOQ. LOD and LOQ are often defined as S/N = 3 and S/N = 10, respectively. In forensic toxicology, these limits are more commonly determined by measuring serial dilutions of an analyte in the matrix of interest and selecting the lowest concentration where measurements meet S/N and additional criteria (3).

Gas Chromatograph–Quadrupole Mass Spectrometer (GC–QMS) and GC–MS–MS

The GC–QMS, also called a GC–mass selective detector or GC–MS, generally requires that analytes be chemically extracted from blood, urine, or other matrices and, in most cases, derivatized to make them volatile prior to introduction into the instrument. A diagram of the QMS is shown in Figure 1 (see page 8A). The GC separates compounds based on differences in volatility and solubility in the liquid, solid, and gaseous phases.

Molecules are ionized as they sequentially enter the ion source. The most common ionization technique is electron ionization (EI). Molecules leaving the GC enter the QMS and are bombarded by a beam of electrons. Electrons are removed from the molecules, producing unstable positive ions (molecular ions) that fracture into more stable fragments. The energy of the electron beam can be adjusted, but it is commonly set at 70 eV. Mass-to-charge ratio and abundances of fragments created by this high-energy interaction are reproducible over time and between instruments and are characteristic for the compound being analyzed. After formation in the ionization chamber, charged fragments transfer with carrier gas molecules into the mass detector that is maintained at lower pressure. Analyte identification is achieved by the detection of a unique fingerprint of ion fragments.

Chemical ionization (CI), often referred to as "soft ionization", utilizes a charged reagent gas (NH_4 or CH_4) to transfer

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charge to a compound. These charged species are more stable than ions formed in EI and fragment less extensively. Both negatively and positively charged molecules can be formed. CI is useful when high sensitivity is required.

The QMS selects ions of specific mass-to-charge ratios and measures their abundances. By creating a complex electromagnetic field inside the four rods, or quadrupole, specific ions are isolated. DC and AC currents (AC current creating a radiofrequency field) are ramped at a constant ratio, allowing only ions with specific mass-to-charge ratios, termed resonant ions, to reach the detector. The QMS can be programmed to scan all mass-to-charge ratios in its mass range, usually 50 to 800 Daltons (Da), or to monitor particular mass-to-charge ratio ions.

The GC–QMS is used routinely to identify and quantify drugs of abuse and licit pharmaceuticals in biological fluids and tissues (4). Typical LOQs are approximately 1 to 10 ng/mL (1–10 ppb) or 50 to 500 pg injected on column. The linear dynamic range is large, usually covering 1–3 orders of magnitude. The ULOQ may be limited by the concentration of internal standard. Internal standards are chemically similar to analytes of interest but can be distinguished by the MS. Addition of a constant amount of internal standard to specimens, controls and calibrators improves the accuracy and precision of measurement. Specimens containing internal standard are analyzed, and a calibration equation is generated with calibrator concentration as a mathematical function of the ratio of analyte to internal standard signal. Analyte concentration in an unknown is calculated by inserting its analyte/internal standard ratio into the equation derived from calibration standards. Deuterated internal standards are preferred because they are the most chemically similar compounds and adequately correct for losses incurred during extraction and other specimen preparation procedures. However, analyte isotope ions often have the same mass-to-charge ratio as the monitored deuterated internal standard ions and can interfere with quantification. This contribution to the abundance of an internal standard ion, if used for quantification, decreases analyte/internal standard ratio and artificially decreases calculated analyte concentration. The relative abundance of isotope ions is small, but the absolute abundance increases with analyte concentration, leading to non-linearity at high concentrations. Increasing internal standard concentration reduces the effect of common analyte ions and elevates the ULOQ, but also increases the LOQ. This is due to the presence of a low concentration of non-deuterated drug in the internal standard. Analysts usually balance these competing factors when selecting the internal standard and its concentration.

Typical inaccuracy and between-assay imprecision for a GC–QMS assay are < 20% and < 10%, respectively. Instruments are rugged, with 20-year-old instruments still in use in some drug-testing laboratories. Instrument life expectancy is usually limited by expiration of software. For confirmation of specimens screening positive by immunoassay, the GC–QMS usually is operated in the selected ion monitoring (SIM) mode. SIM achieves identification by determining the presence of selected ions that are present in specific ratios and quantifies analyte concentration by determining ion abundances compared to those of internal standards. These parameters are compared to those of a single or multiple drug standards. The ratios of abundances of selected ions are unique for a given compound. Although the number of ions monitored can be chosen, three ions and two ion ratios for the native analyte are common for identifying drugs of abuse. The number of ions available depends on the method of ionization. EI produces more ions than CI, thus improving identification but reducing sensitivity. SIM analysis is routinely selected to identify and quantify drugs of abuse in blood and urine (5–9). Similar methods were reported for analysis of potential chemical warfare agents, toxicants of great interest since the escalation of chemical terrorism threats (10,11). In addition, there are new methods for quantifying drugs in oral fluid, hair and sweat (12–24).

Fast GC was developed following GC–QMS modifications including reduced column bore size and more efficient capillary columns, rapid heating rate ovens, and high-pressure carrier gas control. Several recent applications reduce retention times as much as one third, while maintaining acceptable analyte resolution. Applications include analysis of drugs of abuse in urine, 30 different drugs in oral fluid, benzodiazepines in blood, and methadone and its metabolite, EDDP, in blood (25–29).

A GC–QMS instrument is relatively inexpensive and can screen for multiple substances by monitoring common ions. Screening in this manner is ideal for doping control laboratories, where a smaller number of specimens are screened for many drugs or performance-enhancing compounds with an LOD of 1 ppb (100 pg on column). GC–QMS in SIM mode with 10–12 time-acquisition groups of 10–12 selected ions each, is routinely employed for screening urine for anabolic steroids (30,31).

One obvious GC–QMS limitation is its parts-per-billion LOD. With the advent of interest in alternative matrices, laboratories need low quantification limits. For example, typical concentrations of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in hair and oral fluid are, respectively, in the 50 pg/g and 10 pg/mL (ppt or pg on column) range (32,33). To conduct valid quantification in this low concentration range, one must increase the S/N. One approach to reducing noise is two-dimensional chromatography (34–39). A special application of two-dimensional chromatography employs a switching valve with zero dead volume called a Deans Switch® (Agilent Technologies, Santa Clara, CA). This method increases S/N by selectively transferring only a small segment of GC eluent containing analytes of interest to a second column that is coupled to an MS (Figure 2, see page 8A). This eliminates many interfering substances, increasing the S/N. Additionally, a cryotrap can be installed before the second column to concentrate analytes and enhance resolution. A common LOD is 50 ppt (1 pg on column). Figure 3 shows two total ion chromatograms of 1 ng/mL Δ^9-tetrahydrocannabinol in blood, with and without the Deans Switch, to demonstrate improved S/N. New applications with the Deans Switch technology are beginning to appear in the scientific literature (32,40). In addition to improved sensitivity, more stable oven temperatures, and precise electronic control of gas pressures have improved resolution.

Another solution to increasing the S/N is MS–MS, which was proposed over two decades ago (41). Modern MS–MS instruments have LODs less than 1 ppt (42). An ion from the first QMS is directed into a chamber where further fragmentation occurs during collision with gas molecules introduced into the same space. Fragments pass to another QMS for sorting and quantification as described (Figure 4, see page 8A). Although analyte signal is reduced by chamber losses and the presence of fewer ions in the third QMS, noise also is reduced, increasing the S/N.

GC–MS–MS procedures are becoming more common in forensic toxicology laboratories following improvements in vacuum engineering, ion sources, instrument size, and operating software. GC–MS–MS procedures were developed for measuring low concentration analytes (e.g., LSD) in blood or urine (43). New GC–MS–MS methods improve the S/N in critical assays with LODs of 0.1 ppb (5 pg on column), including assays for nerve agent and sulfur mustard metabolites in urine and drugs and metabolites in alternative matrices (44–51).

GC–Ion Trap MS (GC–ITMS) and GC–MS–MS

The ITMS creates a magnetic field within a ring with two endcaps that hold ions in the field until they are sequentially released to the detector (Figure 5, see page 8A). The concept of a quadrupole ion trap was developed many years ago by Wolfgang Paul, who shared the 1989 Nobel Prize in physics for this work (52). In 1983, George Stafford and co-workers developed the technology called mass-selective instability that allowed sequential ejection of ions. This group later refined the ejection process by introducing helium into the trapping volume to reduce the kinetic energy of analyte ions. In this environment, ions of the same mass-to-charge ratio formed packets, making ejection quicker and more efficient, thus improving resolution (53). These innovations allowed ITMS to be introduced into non-research laboratories. Because the instrument traps ions in a three-dimensional chamber, it is sometimes called a 3D-ITMS to distinguish it from ion traps of other designs.

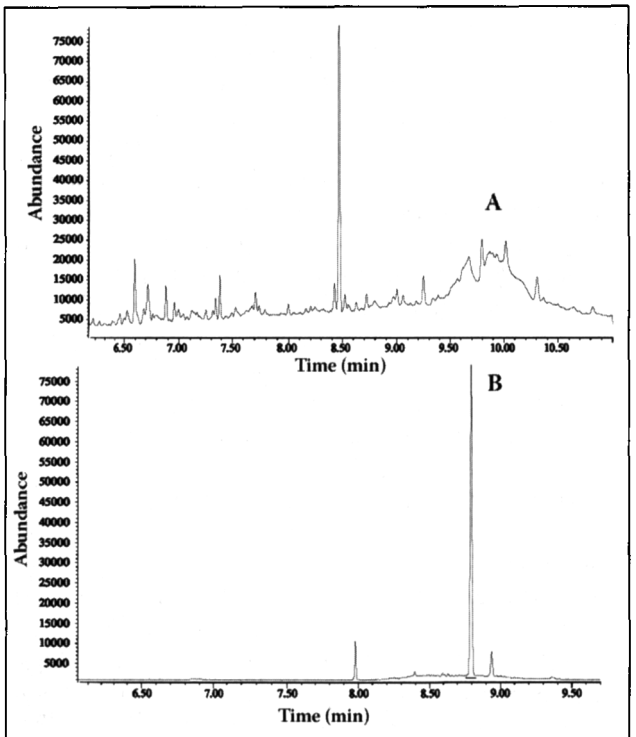

Figure 3. Selected ion chromatograms of 1 ng/mL of Δ^9-tetrahydrocannabinol in human plasma extracts produced without (peak A) and with (peak B) the Deans Switch (Agilent Technologies).

Many of the advantages of an ITMS derive from being able to monitor ions on demand. In contrast, the QMS must analyze ions as they are produced. Ions can be accumulated in the ITMS to improve sensitivity. Ions can be created within the trap instead of externally, and ionization methods are easily changed. Analysts can sort and examine fragments of a principal ion, that is, conduct MS–MS experiments, with a single MS. The ITMS can monitor further fragmentation making MS^n studies possible. Although MS^n with an n of 10 has been reported, typical n are fewer than 4 (54). A disadvantage of the ITMS is that space-charge effects due to too many ions in the trap can occur, causing variability in concentration measurements and ion ratios. In original designs, ionization had to occur inside the trap, and space-charge effects were common. Newer designs allow external ionization to minimize this problem. We will discuss additional methods to reduce concentration-dependent effects later.

Liquid Chromatograph–Mass Spectrometers (LC–ITMS and LC–MS–MS)

In LC methods, analytes are dissolved in a liquid, called the mobile phase, and passed through a column containing small solid particles, termed the stationary phase. Compounds are separated based on differential solubility in the phases. Over the past three decades, improvements in separations have been achieved with solid phases of smaller diameters and consistent particle size and pumps capable of producing high pressure with uniform flow. These technologies are synergistic because higher pressures are required to obtain reasonable retention times when solid phase particles are small. Typical solid phases have particle diameters of 3–5 microns and pressures of less than 34 megapascals (5000 pounds per square inch, psi). Scientists originally described the instrument as a high-pressure liquid chromatograph, or HPLC, and later modified the term to high-performance liquid chromatograph. Recently, solid phases with particle diameters as small as 1.5 microns have become available with operating pressures of greater than 34 megapascals (5000 psi). The instruments are called ultra-performance liquid chromatographs, or UPLC. Compared to a GC, an LC has the advantage of requiring less intense specimen preparation, no derivatization of analytes to improve volatility, and the ability to identify and quantify thermally labile and polar compounds. Common HPLC detectors include ultraviolet-visible spectrophotometers, electrochemical detectors, fluorescence detectors, and MS.

LC–MS and LC–MS–MS techniques in doping control were

recently reviewed (42). Many of the methods also are applicable to drug-testing laboratories. This review is recommended for readers who wish more details of LC–MS theory. To evaluate advantages and limitations of LC–MS detectors, it is necessary to understand ionization techniques.

Development of electrospray ionization (ESI), one of the most common LC–MS interfaces, greatly expanded and improved LC–MS applications. This technology allows transport of charged particles from atmospheric pressure to the high vacuum required for MS (Figure 6, see page 9A). Analytes of interest are charged in solution, nebulized, and desolvated. Solvent evaporates from each droplet until surface tension will not sustain the size ("Rayleigh" limit) and the droplet explodes into many smaller droplets ("Coulombic" explosion). Analyte ions that have been freed from solvent are propelled into the MS by an electrostatic field. Ions are fragmented by collisions with N_2 or similar gases introduced into the reaction chamber, which is termed collision-induced dissociation (CID). ESI interfaces permit MS analyses of molecules in the molecular weight range of drugs of abuse, 50 to 600 Da, or larger molecules, including proteins as large as 232 kDa (55). This ionization method makes it possible to place multiple charges on large molecules yielding lower mass-to-charge ratio ions within the range of MS detection. ESI is the ionization method of choice for polar compounds. The Nobel Prize in Chemistry was shared by John Fenn in 2002 for inventing and refining electrospray ionization techniques (56).

One limitation of ESI is its susceptibility to matrix effects that can cause unwanted ionization suppression or enhancement (57–59). Matrix effects can vary between specimens affecting the relative abundance of ions in the mass spectrum, potentially producing inaccuracy in quantification. Approaches for detecting and evaluating LC–MS matrix effects are available (58–62). Suppression of ionization is greater with increased solvent in the chamber; therefore, an innovation to address this problem simply reduces the amount of mobile phase exiting the column. Nano LC techniques introduce a 100-fold less mobile phase to the ESI interface compared to typical LC systems (63). In addition to reducing solvent, reduction of matrix constituents also diminishes interference. Improvements in analyte identification and quantification include adjusting mobile phase composition to reduce co-elution of matrix components with target analytes and employing matrix-matched calibrators and deuterated internal standards.

Another difficulty with LC–MS and LC–MS–MS is adduct formation. Presence of solvent in the ionization chamber introduces Na^+, K^+, or NH_4^+ that may combine with analyte molecules. These adducts create ions with *m/z* 23, 40, or 18 Da higher than expected. Adducts can contain multiple salt ions and form bridges between ions of differing masses complicating interpretation of mass spectra. Elution solvents that minimize salt ions, for example, formic acid in ultra pure water, are one remedy (64).

Development of searchable libraries for LC–MS and LC–MS–MS remains a problem because fragmentation and resulting spectra differ between instruments. In a review of LC–MS–MS literature, Jansen et al. (65) found that although spectra did not offer expected interinstrument reproducibility, they did have many similar features. Investigators continued to attack this limitation and to report methods for creating reference libraries (66). In a recent report, better reproducibility was achieved by standardizing collision energies to 20, 35, or 50 eV (67). ESI-MS–MS spectra were created for 800 compounds, demonstrating that libraries are now feasible for general use.

Historically, peak capacity, that is, the number of resolved peaks within a defined span of retention time, has been lower for LC–MS compared to GC–MS. Although still true, LC–MS peak capacity has more than doubled with a UPLC that employs solid phase particle diameter < 2 microns and pressures > 34 megapascals (5000 psi) (68). Additional practical applications for biological fluid analyses are available (69,70).

Atmospheric pressure chemical ionization (APCI) is an alternate LC–MS interface with applications for organic compounds in the 50 to 800 Da range. APCI nebulizes solvent and analytes in a manner similar to electrospray technology, but also creates plasma with a corona-discharge needle (Figure 7, see page 9A). In this plasma, proton transfer reactions occur that add additional charge and fragment analyte molecules. Charged particles are electronically filtered before introduction into the MS. In most applications, matrix effects on ionization are less common than with ESI. Adduct formation and variable fragmentation are problematic, as they are for ESI, but can be successfully minimized (59).

Another fragmentation technique available in LC–QMS, LC–ITMS, and LC–MS–MS applications is CID. CID occurs when ionized compounds are accelerated in a fixed area by an electrical charge and collide with neutral gas molecules (N_2, Ar, or He) resulting in fragmentation. CID can occur in-source and in the mass analyzer. In an LC–MS system, in-source CID refers to fragmentation occurring prior to mass detection. By applying a potential difference between the capillary endcap (labeled CID in Figure 6, see page 9A) and the skimmer, molecules are accelerated over a short distance causing them to collide with the drying gas. These collisions result in fragmentation. Increasing the potential difference (fragmentor voltage) increases rates of collision and produces differing degrees of fragmentation. Voltages are optimized to produce desired fragmentation of each ion of interest. Distance between the endcap and skimmer differs between manufacturers and affects fragmentation of a particular molecule at a fixed voltage. In addition, the weight of the collision gas also affects overall fragmentation. A heavy gas, such as Ar, impacts molecules with more energy, causing a greater degree of fragmentation. Also, for some instruments, in-source CID can take place in the octapole region (between the skimmer and MS in Figure 6, see page 9A) by increasing the applied voltage and increasing the number of collisions.

In tandem quadrupole MS, CID occurs in the second mass analyzer (collision cell, Q2 in Figure 4, see page 8A) by increasing the pressure and accelerating ions to collide with gas molecules. Similarly, CID can occur inside an ion trap. Energy is applied to the trap, exciting the ions, which then collide with helium causing fragmentation. Helium also serves to cool and focus the ions inside the center of the trap by forming a buffer between the orbiting ions and the inside walls of the

trap. Mass analyzer CID imparts greater specificity and has a higher efficiency of collision compared to in-source CID. The molecular ions are isolated prior to fragmentation eliminating the possibility of co-eluting ion interferences that can occur during in-source CID. As a result, mass analyzer CID is the preferred method when studying fragmentation patterns and identifying unknowns.

As mentioned previously, one advantage of LC–MS is the ability to identify and quantify compounds without derivatization. Stanaszek et al. (71) described an LC–APCI-MS procedure that identified and quantified eight underivatized amphetamines in hair. Assay performance characteristics were LOD 0.05 ng/mg (50 ppb, 2.5 ng on column), LOQ 0.1–0.3 ng/mg (5–15 ng on column), accuracy ± 16%, and intra- and interassay concentration variations of less than 12% and 19%, respectively (71). This procedure requires minimal specimen preparation and has a large linear range. Methods with similar performance characteristics using LC–ESI-MS are published for eight benzodiazepine metabolites, LSD and metabolites, and several nitrogen-containing drugs (72–74).

Miksa et al. (75) demonstrated the advantage of LC–MS for identifying compounds with differing chemical properties in the same assay. This APCI method screens for acepromazine, ketamine, medetomidine, and xylazine in 0.3 mL of serum or blood with a 2 ng/mL LOD and relative standard deviation of 1–7%. Another LC–MS procedure identified and quantified cocaine, seven metabolites and ecgonidine in blood with an LOD of 0.5 ng/mL, 0.5 ng on column (76). Sklerov et al. (77,78) utilized LC–ESI-MS to rapidly identify and quantify drugs and metabolites in postmortem blood. Examples included identification of overdoses with loperamide (77) and an herbal tryptamine (78). The latter procedure followed a general approach to testing for designer tryptamines in blood and urine using GC–QMS for screening and LC–ESI-MS for confirmation (64).

Analytical methods for quaternary amine neuromuscular blocking agents are needed in forensic toxicology because of their involvement in accidental and intentional deaths. These compounds volatilize poorly and are difficult to derivatize, limiting GC applications. Sayer et al. (79) demonstrated the effectiveness of LC–MS in identifying and quantifying six common neuromuscular blocking agents in blood, serum, urine, and gastric contents. Compounds were quaternary amines with molecular weights in the 500 to 600 Da range, with salts exceeding 1000 Da. An LC–MS–MS method for a similar compound, mivacurium, also has been published (80).

Smith (81) reported an LC–APCI-MS method for VX (O-ethyl S-[2-diisopropylaminoethyl] methylphosphonothioate), an important neurotoxin used as a chemical weapon, demonstrated the usefulness of currently available chiral columns. VX has two important enantiomers. The isomer with a (–) phosphorus chiral center is an order of magnitude more toxic than the (+) isomer. Using a Chiracel OD-H column (Chiral Technologies, Exton, PA), both isomers were separated, identified and quantified in human plasma (81). Similar procedures have been reported for metabolites of nicotine in plasma and *d,l*-dextromethorphan in regulated formulations (82,83).

LC–MS–MS applications continue to be developed to handle difficult biological matrices and to improve specimen throughput. Herrin et al. (84) examined postmortem blood, a matrix that typically has many interfering substances. The presence of over 400 compounds could be screened using an ion trap LC–MS–MS system with LOD as low as 5 µg/L (5 ng on column). Murphy and Huestis (85,86) developed an assay for analyzing drugs and glucuronide metabolites in the same analysis; morphine and codeine analytes were quantified in human urine and buprenorphine and metabolites in human plasma. Fourteen drugs that were substrates for CYP3A4 were identified by Racha et al. (87) using a high throughput LC–MS–MS with an enzyme inhibition assay for detection. Rule et al. (88) combined high throughput microtiter well systems that employed solid-phase extraction prior to LC–MS–MS analysis. Nordgren et al. (89) screened for 23 analytes in urine by direct injection LC–MS and suggested that large volume laboratories could replace immunoassays with LC–MS screening to reduce false-positive test results.

There also are other specialty applications for LC–MS–MS. In addition to product ion scanning, described in the GC–MS–MS section, one can scan ions in the first QMS and monitor only one ion in the third QMS (Figure 4, see page 8A). This method, termed precursor ion scanning, allows identification in the first QMS and quantification using a single ion in the last QMS. A different application includes capturing spectra of unidentified synthetic steroids in urine, for which there are no reference standards, followed by monitoring a product ion that is characteristic of steroid molecules to assist in identification (90). Choo et al. (91) utilized LC–APCI-MS–MS for analyzing drugs in meconium, a matrix containing many endogenous interferants. Other applications include characterization of glycopeptides and lipids (92–94). Neutral loss scanning is a less common technique but has been helpful in monitoring glucuronides and lipids (93,95). In the neutral loss scan mode, a spectrum is obtained that shows all the precursor ions that lose a neutral moiety of selected mass (Figure 4, see page 8A). In a recent LC–MS–MS assay, investigators quantified nine metabolites of cocaine and heroin in a single analysis using selected reaction monitoring (SRM, also called multiple reaction monitoring) (96,97). SRM methods monitor specific precursor to product ion transitions (Figure 4, see page 8A). The ion transition is relatively unique for a specific compound and monitoring a single product ion versus multiple fragments improves quantification. Coles et al. (98) reported a similar SRM method that allowed for the simultaneous LC–MS–MS analysis of six opiates in urine, serum, plasma, blood, and meconium. Compared to GC–MS, this method increased specificity and avoided glucuronide hydrolysis.

A linear or two-dimensional (2D) version of the quadrupole ion trap was described in 2002 (99). The quadrupole arrangement of this trap is similar to the QMS described earlier (Figure 8, see page 9A). Ions enter the trap through the opening in the front and traverse back and forth along the z-axis, trapped by a radial oscillating electric field (radio frequency). The ions are propelled into the trap and their kinetic energy reduced (cooled) by interactions with gas molecules such as helium introduced as was discussed in the GC–ITMS section. When the operator lowers the trapping potential, that is, the mass-se-

lective instability technique, ions are radially ejected with a narrow kinetic energy range, as low as ± 0.4 eV. Radial ejection is important to reduce unwanted energy transfer from motion along the z-axis. The trapping potential change also can be regulated to produce pulsed ejection of ions. A common instrument for analyzing ejected ions is a time-of-flight (TOF) MS. As discussed later, the nearly monoenergetic ions entering the TOFMS improves mass accuracy. Unlike the 3D-ITMS, the 2D-ITMS can be continuously filled with ions while other ions are ejected in pulses from the radial port. Compared to a 3D-ITMS, the 2D-ITMS has a 15 times higher ion capacity, 3 times faster scan rate, 100% versus 50% detection efficiency, and 70% versus 5% trapping efficiency (99–101). Mayya et al. (100) compared 2D-ITMS with 3D-ITMS using over 100,000 MS–MS spectra acquired with identical complex peptide mixtures and common acquisition parameters. More than 70% of the doubly and triply charged particles, but not the singly charged particles, had better quality of fragmentation spectra. They concluded that the number of peptides and proteins identified increased four- to sixfold when using the 2D-ITMS.

Other LC–MS–MS methods published in the past three years include opiates and cocaine in oral fluid (102); ricinine in urine (103); sulfur mustard metabolites in serum (104–106); poly-chlorinated hydrocarbons in plasma (107); nalmafene in plasma (108); butadiene and metabolites in urine (109); risperidone and its 9-hydroxy metabolite in plasma (110); quetiapine in plasma (111); buprenorphine and norbuprenorphine in serum (112); and 26 benzodiazepines and metabolites, zolpidem, and zopiclone in blood, urine, and hair (113). LOD for these methods was about 0.1 ppb (10 to 250 pg on column). Many of these compounds are typically found in low concentrations and are difficult to analyze by GC methods. Benzodiazepines, zolpidem, and zopiclone are often used by perpetrators in drug-facilitated sexual assault and have low concentrations in blood and urine collected from the victim days after ingestion. Investigators often obtain specimens in this delayed timeframe and want the longest detection times possible. In the last study cited, an LC–MS–MS assay allowed analysis of multiple matrices with detection times for urine of greater than 14 days (113,114). With low detection limits, hair analysis may be able to detect single doses of some of these drugs and improve a police investigator's ability to identify single ingestion of a substance used for drug-facilitated sexual assault (115–117).

One method to reduce unwanted ionization effects in LC–MS is to reduce specimen volume. Of course, this also reduces analyte abundance and, therefore, signal strength. New ion trap LC–MS–MS systems with lower detection limits make possible the detection of small amounts of sample on column. Tomkins et al. (63) reported a chip-based nanoelectrospray MS–MS, SRM mode, method that could detect 0.49 ng cotinine, a metabolite of nicotine, in 1 mL oral fluid. The chip is the LC system, and the amount of specimen extract on the chip was 10 µL, providing an LOD of 4.9 pg on column. The assay was linear to 40 ng/mL, an important feature, because cotinine has a large expected concentration range. This nano LC method also employed a 96-well microtiter plate to reduce labor and increase throughput. In this preliminary study, accuracy and precision results were not reported, and it was noted that despite the small injection volume, occasional specimens had reduced signal that was possibly due to ion suppression from oral fluid components. The investigators also found that some micropipet tips or ESI nozzles were blocked by solid material in the specimen. These problems will need to be resolved by future innovations, but the concept of a system with small specimen size, low detection limits, multiple drug testing platforms, and high throughput is promising.

Magnetic Sector MS and GC–Combustion Isotope MS

A magnetic sector MS sorts ions by passing them through a channel in a magnetic field. An ion's route of travel curves in the constant magnetic field and the arc of the curve is a function of ion mass-to-charge ratio. The detector sorts ions with different mass-to-charge ratios and makes accurate measurements. Using peak matching or dynamic voltage scanning techniques, ion masses can be measured in 10^{-3} to 10^{-5} Da increments compared to 10^{-1} to 10^{-2} Da increments by typical QMS or ion trap instruments. The instrument's resolution can exceed 100,000 compared to less than 10,000 for a QMS (resolution = ion mass divided by the smallest measurable mass difference) and mass measurement accuracies of less than 10 compared to 100 ppm (118). The more precise molecular masses provide improved elemental composition yielding better discrimination between unknown substances. Instruments can be expensive but have applications in compound identification and sport testing.

A GC–isotope combustion MS is a special type of magnetic sector instrument that accurately measures the mass of combustion products. As the name implies, the technique separates compounds and measures the ratios of stable isotopes of carbon, nitrogen, and oxygen in combustion products; CO_2, H_2, N_2, or CO. Isotope ratios of the original molecules can be calculated from these data. In general, this technique is useful because compounds have different isotope ratios of elements that compose them based on origin of precursors. For example, exogenous testosterone has a lower $^{13}C/^{12}C$ ratio than endogenously produced testosterone; this ratio can be used to distinguish testosterone doping in sports (119,120).

The GC separates volatile compounds in the same manner described. Carbon and nitrogen compounds eluting from the column are directed into a combustion reactor with a carrier gas, usually He. The compounds are oxidatively combusted when passing over a Cu/Ni/Pt wire maintained at greater than 900°C. Combustion products enter a reduction reactor with Cu wires at 600°C to reduce nitrogen oxides to nitrogen. Hydrogen and oxygen products are usually prepared in higher temperature reactors. Even though novel applications with the latter two elements are reported each year, they are less common in forensic toxicology. After water is removed in a separator, the abundances of combustion products of different masses are measured in a single magnetic sector instrument. As an example, the abundances of masses 44, 45, and 46 Da are

measured for the stable isotopes of carbon corresponding to $^{12}C^{16}O^{16}O$, $^{13}C^{16}O^{16}O$, and $^{12}C^{16}O^{18}O$, respectively. Isotope ratios are calculated and isotope ratio difference (δ) is expressed as

$$\delta\ ^{13}C\ (‰) = \frac{[(^{13}C/^{12}C)\text{specimen} - (^{13}C/^{12}C)\text{standard}] \times 10^3}{(^{13}C/^{12}C)\text{standard}}$$

Units are dimensionless and termed "per mil" (‰). Differences in isotope ratios are small and must be measured accurately. Compounds from two different origins are contrasted by comparing the δ value for each. Special care must be taken to reduce isotopic effects from co-eluting compounds and derivatization moieties, especially if they contain a large number of the elements being monitored. This requires baseline separation of compounds and selection of derivatization methods with reproducible reactions. For analytes such as steroids, LOD are usually 2–5 ng on column, ULOQ 100–200 ng on column and coefficients of variation < 2% (119).

There have been many studies to determine the origin of steroids and drugs in human fluids utilizing isotope ratios (119,121,122). Aguilera et al. (119) found that δ ^{13}C for two endogenous androstanediols in urine were in the ± 3SD range of –22 to –28‰, whereas the values for athletes taking supplemental testosterone were near –30‰, indicating that this method could be used to identify illicit use. Similar studies showed that GHB in human postmortem blood differed from synthetic GHB, indicating that the method could distinguish ingested from endogenous GHB in forensic cases, such as drug-facilitated sexual assault (123). Abramson et al. (124) recommended that investigators administer drugs containing stable isotopes in pharmacokinetic studies to allow scientists to distinguish current administration of drugs from previous use or endogenous production. The country of origin for seized heroin was identified using carbon isotope ratios (125). The investigators found that the δ ^{13}C (‰) for compounds in heroin from North Korea were distinct from those found in Southwest Asian, Southeast Asian, South American, and Mexican heroin. The fact that geologists have used stable isotope methods for years suggests that more applications for human toxicology are possible even though expected δ values are small (126).

Time-of-Flight Mass Spectrometers

A TOF-MS accurately determines the mass-to-charge ratios of ions by measuring flight time after acceleration in a vacuum tube by high voltage. Ions are created by electron bombardment, ejected using pulsed electrical potential, usually with pulses of 1–10 ns, and electrically focused to give all ions the same energy before being accelerated in a flight tube. Mass resolution and accuracy can be similar to those achieved with magnetic sector instruments. Resolution has improved in modern TOF-MS instruments by including an ion reflector at the end of the flight tube that bends the ion path back toward the entrance before detection. This reflection compensates for the different flight distances of ions with the same mass-to-charge ratio but different entrance kinetic energies. Ion paths on the order of 2 m with flight times of 5–100 μs allow rapid, accurate time measurements. Advantages of this method are short analysis times, accurate mass measurements and large dynamic ranges, up to 500 kDa. Instruments are compact, unlike original models, and prices are in the range of US$200,000.

Ojanpera et al. (127) used an LC–TOF-MS for qualitative identification of compounds in urine by accurate mass measurement. One can determine the elemental formula from molecular mass, and interpretation usually is unambiguous if mass resolution is greater than 10,000 and molecules are less than 300 Da and contain C, H, N, and O. When mass-to-charge ratio increases, finding a unique formula may require higher resolution and accuracy near 10 ppm or less. This technique distinguished compounds of 100 to 400 Da by measuring masses with accuracies of 5–10 ppm. Investigators reported routinely reducing choices to two or three unknown substances using a library of 7640 compounds without considering LC retention times. Typical drugs found in postmortem specimens were easily identified. Pavlic et al. (128) used similar techniques with mass-spectral libraries to identify over three hundred different drugs. In another application, glucuronide metabolites of ketobemidone, a postoperative analgesic, were identified by CID in an LC–MS–MS. Identification was improved by accurate mass measurements in LC–ESI-TOF-MS instruments (129). Absolute mass errors were in the range of 1.4 to 2.1 ppm for most metabolites and 4.2 to 6.9 ppm for ketobemidone glucuronide.

Cocaine and ecgonine methyl ester in rat plasma were quantified using an LC–TOF-MS (130). A typical LC–TOF-MS can acquire 20 scans per second compared to 2–5 scans per second for a QMS. Coefficients of variation for concentrations of 50 repeated injections over 5 days were 0.58% and 0.73% for the two drugs, respectively. LOQs for cocaine and ecgonine methyl ester were 0.5 and 5 ng/mL (12.5 and 125 pg on column). ULOQ and coefficients of variation for assay control samples were 10,000 ng/mL and 3.7–9.3%, respectively. Clauwaert et al. (131) reported a similar procedure for cocaine and metabolites in oral fluid. Others quantified diazepam, nordiazepam, and bromazepam in blood by a GC–TOF-MS method with an LOD less than 1 ng on column (132). LOD for a GC–QMS in full scan mode for the same specimens ranged from 5 to 34 ng.

Analysis of proteins and other macromolecules is important in toxicology, but has always been difficult, for example, identification of ricin, a toxic RNA translation inhibitor (MW = 66,000). Macromolecules are not easily manipulated in MS vacuum chambers and are often beyond the MS measurement range. Part of the impetus to develop MS methods for identification and quantification of macromolecules, in addition to acute toxic substances, was a requirement to test for performance-enhancing proteins in competitive sports, such as human chorionic gonadotropin, growth hormone, insulin, erythropoietin, and synthetic substitutes (42). Innovations that allowed MS analysis of macromolecules included placing multiple charges on substances with ESI techniques to bring the mass-to-charge ratio within the detector range (133,134). TOF-MS methods cited here use ESI ionization (127,129, 130,132).

Another ionization method is matrix-assisted laser desorption

ionization (MALDI) with TOF-MS detection. For this technique, a solution containing an unknown analyte (about 10μM) and a charge-transfer matrix chemical, such as 2,5-dihydroxybenzoic acid (about 10mM), is evaporated onto a plate, usually made of stainless steel. A laser beam (often an N_2 laser with wavelength 337 nm) is directed at the film of protein. For some applications, the analyte is deposited on the plate from the effluent of a nano LC. The laser is tuned to place the protein in an energy state that allows it to incur multiple charges in an electric field. The result is a mass-to-charge ratio in the measurement range of a TOF-MS. Advantages of the MALDI-TOF-MS are low LOD for macromolecules, ability to reanalyze the same analyte mixture from a stored plate, and enhanced data acquisition at various points along a chromatographic peak. Disadvantages are that continuous flow monitoring is not practical and matrix interference for small molecules is greater than those of competing techniques. Quantification also is difficult, unless one uses internal standards that are often unavailable.

Whiteaker et al. (135) identified and quantified the heme moiety (*m/z* 616.5) in 0.5 mg bacillus (Anthrax) spores using a MALDI-TOF-MS. An LOD of 200 fmol heme was achieved. The LOQ was higher, 400 fmol, due to ion suppression. Deuterated heme was unavailable. Investigators used the structurally similar compound cobalt (III) protoporphyrin as an internal standard and reported coefficients of variation of 7% to 11%. In an investigation of the mechanisms for incorporation of drugs in hair, investigators identified four in vitro amphetamine-melanin adducts using MALDI-TOF-MS. Identification was accomplished with deuterated analogues of precursors. Accurate masses of the smallest and largest adducts were within 14 and 70 ppm of theoretical masses, respectively. Ion suppression can be a problem with a MALDI-TOF-MS as investigators demonstrated in attempts to forensically identify variants of ricin in different castor bean preparations (136,137). Low molecular mass components caused ion suppression and molecular ions did not consistently form for ricin, MW ≈ 64,000 (136). Variant ricin proteins were subsequently identified by characteristic peptide profiles in castor bean digests.

Fourier Transform Ion Cyclotron Resonance Mass Spectrometers (FTMS) and Orbitrap™

An FTMS is the ultimate instrument for high mass resolution (up to 10^6) and accuracy (1 ppm), but is infrequently found in toxicology laboratories because of the high price, usually US$800,000 to $1,000,000. FTMS testing is becoming more popular as specialty laboratories contract services offering testing at an affordable cost. Current customers are primarily scientists working in proteomics; however, forensic toxicology applications are being published (127). An FTMS can be used in toxicological analyses as a detector for GC, LC, and capillary electrophoresis (CE) instruments or as a standalone analyzer. The technique has advantages in studies requiring exact mass measurements and analysis of macromolecules.

A brief theory of an FTMS is presented in Figure 9 (see page 9A). Following ionization, ions are trapped in a chamber, called the Penning trap, by an electric and 3–9 Tesla magnetic field. Ions can be visualized as spinning in a circle in a plane perpendicular to the magnetic field at an angular frequency (velocity divided by orbital radius) that is called the ion cyclotron frequency, which is inversely proportional to the mass-to-charge ratio. This initial frequency is difficult to measure for two important reasons. First, the orbital radius is small and ions do not travel close enough to detection plate electrodes to induce a measurable current. Also, ions produced outside the FTMS enter the Penning box at various angles, and in reality, are not all in the plane perpendicular to the magnetic field. They are in a variety of positions and planes and many charge effects they exert on detection plates cancel each other. For these reasons, energy is added from excitation plates to increase ion cyclotron frequency and to place ions in the same orbital phase. This is accomplished by an electric field, that is, potential difference between excitation plates, that has a constant absolute magnitude and polarity oscillating near or at the ion cyclotron frequency (i.e., the excitation and ion cyclotron frequencies are in resonance). The packet of ions within the observed mass-to-charge ratio range absorbs the excitation energy, giving ions the same larger orbital radius and making them spatially coherent. When ions pass near detection plates, they induce a charge in the molecules that compose the plate. The amount of charge induced is proportional to the charge of the ion divided by its distance from the detection plate. Because ions are moving and distance from the detection plate varies with time, the induced charge also varies with time. The change in charge with time, also called detection current or, more appropriately, detection frequency, is the signal that is measured and is a function of the charge and mass of the ion.

As an example, consider a 100 Da positive ion of single charge at room temperature in a uniform 7 Tesla magnetic field. The ion cyclotron radius would be about 0.03 mm. If one applied a constant electric potential between excitation plates 2 cm apart oscillating at ± 1 volt for 1 ms, the orbital radius would increase to 0.72 cm and all ions would be orbiting coherently. This excitation energy is easily achieved and markedly improves detection. Detection signal is a function of the frequency of the induced oscillating current, but can be mathematically converted to a function of mass by Fourier transformation, named after the French mathematician who first described the mathematics in 1822. Measured frequencies are typically in the range of kHz to MHz and can be measured with accuracies to seven significant figures. Using calibration standards, masses also can be determined through transformation with similar accuracies.

In practice, the excitation current is pulsed for a short period of time, usually milliseconds, and the detection pulse signal also is present for a short time period. One of the many advantages of FTMS is that ions of many different mass-to-charge ratios can be present in the trap and signals detected simultaneously for each. Events can also be sequenced to allow MS^n operations. FTMS have theoretical LOD of one attomole (10^{-18} mole) and a reported LOD for carbonic anhydrase (MW = 28,780.6 Da) of 9 attomoles, or 0.26 pg (138,139).

Mass can be measured accurately to 1 ppm and resolution of 10^6 is possible. Because the induced charge in the detection plates is directly proportional to charge on the ion, multiply charged ions will produce a larger signal. This gives FTMS advantages for examining macromolecules with multiple charges. Another advantage of the FTMS is that excitation increases the kinetic energy of ions and facilitates fragmentation when these ions collide with a neutral carrier gas introduced into the chamber for CID (138,140). For example, the kinetic energy of the 100 Da ion in the conditions mentioned above would be 1200 eV. After collision, this high-energy ion would fragment extensively and the fragments could be examined to characterize the molecule and also to perform MS^n experiments.

There are limitations. Detection and mass accuracy are limited by interactions between like-charged particles in the confined space, so-called space charge effects mentioned in the GC–ITMS section. Space charge effects are the principal interferences, but others include limitations in shielding from external electrical signals, field distortions due to the finite compartment length, and variations in magnetic field strength (141). To counter space charge effects, low pressures are maintained inside the trap, usually around 10^{-9} torr. The low pressure requires long pumping times, especially after collision or cooling gases have been introduced into the trap to conduct experiments. Additional details can be found in recent reviews (138,142).

FTMS has served as a primary reference method for validating other methods (118,127,143). Ojanpera et al. (127) reported a new LC–TOF-MS method with 20–30 ppm mass accuracy and compared this to an LC–FTMS with a mass accuracy of 3 ppm for the same set of urine specimens. Quinton et al. (144) analyzed the venom of the mollusk, *Conus virgo*, by FTMS using both nano LC–ESI and LC–MALDI and found 64 distinct masses in the 500–4500 Da range. Two major components were identified as conotoxins based on masses of 1328.5142 and 1358.5592 Da. Similar studies of snake venom by LC–MS-FTMS demonstrated that the methods gave important information but companion techniques were needed to interpret the complex results, primarily because of the lack of appropriate databases (145,146). In these and other studies, investigators found that peak areas of macromolecules in biological fluids varied less than 10% within and 30% between runs (145–147).

Wang et al. (147) examined proteins in rat kidney homogenates and human plasma using an LC–MS–MS system with a FTMS detector followed by five MS–MS scans using a 2D-ITMS detector. They achieved reproducibility similar to the three previous studies and linear response over a range of concentrations for multiple analytes. These studies in proteomics demonstrated the power of LC–MS–MS systems for examining macromolecules, a relatively new method for forensic toxicologists. Recent studies of steroids in biological fluids, cytochrome c, and metabolic profiles have been reported using FTMS (148–150). The nanochip ESI technology described above also was coupled with FTMS to produce an accurate mass screening system for glycoproteins; mass deviations < 6 ppm (151). Additional reviews for application of FTMS in other fields are available (152,153).

We mentioned the disadvantage of long pumping times for CID studies using FTMS. One solution is a hybrid system that links a 2D-ITMS with FTMS. CID is conducted in the 2D-ITMS trap that does not require a 10^{-9} torr vacuum (154,155). Pressures within this first trap, 10^{-5} to 10^{-6} torr, are more easily achieved and maintained. A number of studies can be performed in the 2D-ITMS, including full scans in seconds, and subsequent accurate masses determined for ions transferred on demand to the FTMS.

In 2005, the Orbitrap (Thermo Electron, Bremen, Germany), a new innovation in MS, was released. The principle of the Orbitrap is similar to the FTMS except that the magnetic field is replaced with an electric field that produces a similar ion trapping function. As with FTMS, the instrument can collect information from many ions at once or focus on one ion at a time allowing MS^n operations. One advantage of the Orbitrap is that it is less expensive, typical costs are about US$600,000, and there is no requirement to maintain a high field strength magnet. Some published studies using this technology reported resolution of 60,000, mass accuracy of 2 ppm with internal standards and real-time acquisition of three MS–MS spectra per second (156,157).

Cyclic Voltammetry and Pulsed Electrochemical Detectors (PED)

Cyclic voltammetric systems can be used as LC detectors. They are not common in forensic toxicology laboratories but have been used to identify drugs in biological fluids and have advantages in special applications. For example, glucuronides that are common metabolites of drugs and poisons are poor chromophores but are amenable to electrochemical detection. Electrochemical detection identifies compounds in solution by measuring electric current as a function of potential difference between electrodes in contact with the analyte solution. A compound's reduction potential is relatively unique and when an increasing potential difference between electrodes in solution reaches this voltage, current will increase as the compound is reduced at the electrode's surface, thus providing a means of detection. For continuous monitoring, the compound in solution must be replenished at the electrode as molecules are reduced. In some systems this is accomplished by stirring the solution. Cyclic voltammetry solves the problem of reduced compounds on the electrode surface by oxidizing them. The full testing cycle progresses as follows: a potential difference between electrodes is increased, the compound is reduced on the cathode surface at its reduction potential, and an increase in current is observed. The instrument continues to increase voltage to a selected limit. At the limit, the polarity is reversed, the cathode becomes the anode, and voltage increased again. The compound is oxidized as the voltage reaches the oxidation potential (equal in magnitude to the reduction potential). The analyte is identified by the unique voltage required to reduce and oxidize it. The analyte concentration, which is proportional to the amount of current recorded, is determined by

comparison to standards. This technique avoids the problem of depletion of analyte at the electrode surface and also ensures that all molecules are in the same electrochemical state on the reverse voltage ramp. Many biogenic amines have unique reduction potentials and have been analyzed in biological fluids by cyclic voltammetry (158,159). Modern applications range from screening of antioxidants in pharmaceutical preparations to in vivo monitoring of dopamine in neuroscience research (160–162).

A problem with electrode measurements is that the electrode surface can become fouled, producing interference in current measurements. One solution is a PED, an electrochemical device operating in a mode that applies a pulse program to a noble-metal electrode, producing continuous surface regeneration. PEDs are useful for detecting compounds that are poor chromophores and therefore not easily detected by spectrophotometry. PEDs are not as specific as MS detectors, but much less expensive. Typical LOD for LC–PED are 100–500 pg on column with concentration intra-assay coefficients of variation in biological fluids of less than 15%. Even though electrodes are not readily fouled, electrode surfaces do eventually deteriorate and need to be changed when analysts observe diminished signal strength of quality control samples. PEDs have been used to analyze pharmaceutical preparations with hydroxyl and sulfur-containing organic compounds, explosive-containing environmental specimens, and enantiomers (163,164). Kaushik et al. (165) identified and quantified ethyl glucuronide, a direct metabolite of ethanol used to assess drinking status, in human urine; the authors proposed a generalized approach using PED assays to analyze drug glucuronides (166). As part of a study comparing various electrodes for PED, Cataldi et al. (167) reported a method for identifying iodide in urine, a test useful for proving adulteration in drug-testing programs.

Capillary Electrophoresis

CE is experiencing a resurgence in toxicology laboratories because it offers some advantages over chromatographic methods (168,169). The instrument separates compounds based on electrophoretic mobility and interaction with a liquid or solid phase adsorbent instead of the chromatographic mechanism of phase solubility. The basic design of the instrument is a fused silica capillary connected to buffer at one end and normally the same or similar buffer at the other end. A high electric potential difference is placed across the tube creating electroosmotic flow of buffer as shown in Figure 10. Analytes (neutral compounds, anions, and cations) are introduced into the tube at the source end and flow with the buffer at different rates toward the detector, usually the cathode. Although anions are attracted to the anode, they move toward the cathode due to the greater magnitude of electroosmotic flow. Other factors that influence migration are the number of charges, size, and mass of the analyte. CE offers advantages including requirement of only small volumes of buffer and specimen, analysis of basic, neutral, and acidic compounds in the same assay, ready determination of enantiomeric purity, and the capability to analyze large, water-soluble biomolecules (170). Unlike HPLC, there is no transfer of analyte molecules between mobile and stationary phases, increasing the theoretical plates for CE. As analyte migrates, it also is compacted by the same force that caused electroosmotic flow. The result is narrow, discrete bands of analyte at the destination end of the capillary, the location of the detector. Detectors include UV, visible, and fluorescence spectrophotometers; PED; and MS. Types of MS and interfaces are similar to those used in LC. Many CE performance characteristics depend upon the detector selected.

Reviews of CE have been published in many fields of science including forensic toxicology (168,171–173). CE methods have been utilized for identifying and quantifying a wide variety of drugs and biological compounds (174–176). Lurie et al. (169) reported application of CE for analysis of a variety of controlled substances in a single injection that separated acidic, neutral, and basic drugs. The instrument was inexpensive, simple in design, easy to maintain, and used very small amounts of specimen and buffer. Sensitivity of CE varies over a general range of milligrams per liter (parts per million) for detectors such as direct UV absorption to micrograms per liter (parts per billion) for conductivity and laser-induced fluorescence detectors (176,177). Derivatization and sample concentration enhanced sensitivity (172,177–181). Chiral separations using CE methods also have been reported (170–173,178, 180,181). Unlike LC or GC methods, which require specialized columns, CE chiral separations can utilize more cost effective additives, such as cyclodextrins, to the mobile phase (182). Additives enhance separation depending upon the analytes' distribution coefficients with a particular cyclodextrin. Innovations in dynamically coated capillaries promise to improve resolution of many different compounds and expand applica-

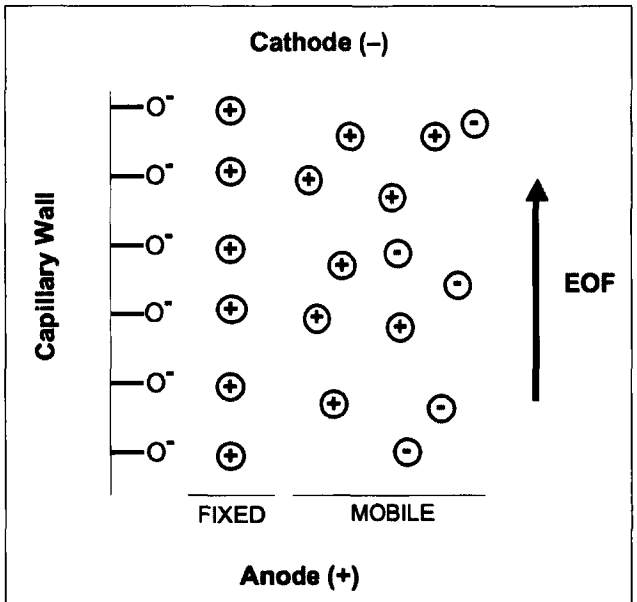

Figure 10. Schematic of a capillary electrophoresis instrument. Cations, anions and neutral molecules are propelled toward the cathode by electroosmotic flow (EOF) of the buffer. They are separated based on electrophoretic mobility and interaction with the adsorbent before entering a detector positioned at the cathode end of the capillary.

tions, such as accurate determination of pK_a values for drugs (169,183,184). MS detectors improved certainty in identification (173,185). The small amount of compound exiting the capillary is one limitation of a CE–QMS (ppm LOD) compared to an LC–QMS (ppb LOD). Despite this limitation, microchip technology using CE is becoming more common (186), and recent sample enrichment methods for CE–MS have lowered limits of detection (187). Precision in CE migration times can be variable under some conditions and may be reduced by mobile phase additives (188).

Conclusions

Two Nobel prizes were awarded for novel MS technologies with applications in forensic toxicology: one to Wolfgang Paul for the quadrupole ion trap mass spectrometer and another to John Fenn for electrospray ionization methods. The QMS, ITMS, and LC–ESI-MS are important instruments derived from these innovations. With these tools, toxicologists routinely identify and quantify a range of compounds in biological fluids from small molecular weight drugs, steroids, and toxins to 232 kDa macromolecules.

Recent refinements such as better vacuum pumps and novel ion sources for GC–MS have improved ruggedness, a necessary requirement for analyzing biological specimens such as blood, hair, and soft tissues. Some GC–QMS instruments, for example, are still operating in high-volume laboratories after two decades of use.

Innovations have reduced the time required for analysis. Examples include reduced column bore size, high heating rate ovens, high pressure carrier gas control, and more efficient capillary columns leading to fast GC methods. Software design has made LC–MS and LC–MS–MS operation easier, faster, and within the abilities of less experienced laboratory analysts. The development of ESI and APCI interfaces expanded LC–MS applications and improved reliability of quantification. New additives to mobile phases and dynamic coatings improved resolution and precision in CE. Some laboratories have transitioned from chromatography to CE methods to reduce retention times and analyze mixtures for acid, neutral and basic compounds in a single run. The 2D-ITMS with monoenergetic expulsion of ions combined with better ion reflectors in TOF-MS have resulted in greater mass accuracy measurements. These modern instruments identify compounds by mass spectra that are collected in seconds compared to minutes for more traditional MS systems. One promising application is identification of unknown compounds for which there are no reference standards using exact mass measurements.

The quest to achieve lower limits of detection led to developments in both chromatography and MS. The Deans Switch, a two-dimensional chromatographic technique, allows analysts to select eluents from a GC column in small segments of time. This reduces interferences from unwanted compounds eluting just prior to the retention time of interest and also preserves the MS detector. Modern MS–MS, after two decades of improvement, are smaller, more rugged, and have more user-friendly software compared to predecessors. These improvements have made MS–MS more useful in forensic toxicology laboratories, especially when methods requiring an LOD of less than 1 µg/L (1 ng on column) are needed, such as with hair, oral fluid, and sweat testing analyses. Of course, the ultimate instrument for a low LOD, as well as mass accuracy and resolution, is the FTMS. One publication documented an LOD of nine attomoles, or 0.26 pg (139). The instrument has greater sensitivity for multiply charged ions making it ideal for examining macromolecules.

Hybrid instruments are one tool to help analysts address the expanding requirements of forensic toxicology laboratories in criminal investigations. An LC–2D-IT-TOF-MS linked to a FTMS allowed investigators to collect identifying information from CID experiments in the first MS and then capture, on-demand, exact mass information for selected ions in the FTMS. Applications for this type of versatile instrument are promising. Improvement of LC interfaces, columns, flow rate pumps, and operating software have made LC an option for non-research forensic toxicology laboratories. Now with acceptable precision, resolution, and quantification, analysts can exploit the advantages of an LC–MS over a GC–MS by examining biological fluids for nonvolatile compounds. The quest to routinely examine drugs and glucuronide metabolites in the same analysis may be realized (189).

Although achievements in analytical toxicology are many, limitations remain. LC–MS spectra are not reproducible between instruments making it difficult to compile common spectral libraries. Standardization of collision energies is addressing this limitation, but compilation of spectral libraries is in its infancy. Ion suppression remains a problem for some matrices and applications. To achieve peak capacity equal to GC–MS, higher pressures are needed and some recent commercial instruments and columns routinely achieve 103 megapascals (15,000 psi). For equivalency in separation of analytes, however, Medina et al. (68) calculated that about 620 megapascals (90,000 psi) would be needed. This high pressure may not be practical. Another solution would be new innovations in columns, mobile phases and high temperature methods that could enhance separation. Microchip methods show promise but practical problems with clogging of narrow channels and sporadic ion suppression limit expansion to routine testing. The need in CE applications is a more sensitive detector. There are a number of methods published for CE–MS, but more sensitive and robust procedures are needed before this instrument can reach its potential in forensic toxicology laboratories.

The biggest limitations for FTMS are its high cost and requirement of ultra low vacuum. The Orbitrap, a new technology introduced in 2002, may remedy some of the problems by measuring accurate masses at a lower cost and with fewer maintenance requirements.

The authors hope that colleagues will pursue and discover new innovations to overcome limitations of our current analytical methods and that these innovations will be applied to the analysis of an expanded inventory of analytes and matrices in forensic toxicology laboratories. Improving instrument ruggedness, increasing throughput, lowering LOD and LOQ,

and developing versatile systems to expand applications will help us meet future analytical challenges.

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