Chapter 2

LC-MS vs. GC-MS, Online Extraction Systems, Advantages of Technology for Drug Screening Assays

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Abstract

This chapter reviews recent applications of mass spectrometry to systematic toxicological analysis (STA), where extended lists of compounds of toxicological interest are screened, as well as to the general unknown screening (GUS), where all exogenous compounds present in a sample are tentatively detected and identified, without any preselection. Many recent improvements in sample preparation, chromatographic separation, gas chromatography-mass spectrometry, and above all liquid chromatography-mass spectrometry techniques are described, which are applicable or have been applied to STA and/or GUS, generally with promising results. These improvements come from miniaturization and automation of solid-phase extraction, turbulent-flow or ultrahigh-pressure liquid chromatography, linear ion traps, accurate (e.g., time of flight or orbital trap) mass spectrometry, as well as software refinements to alternate between different ionization modes or automatically interpret the results. It also shows that robust LC-MS/MS techniques already exist for STA or GUS, which are at least as efficient as the traditional techniques used in most toxicology laboratories, such as GC-MS or high-performance liquid chromatography with diode-array detection, as shown by three comparative studies. However, the major drawback of LC-MS/MS in the full-scan mode for STA or GUS is that it still lacks universal reference libraries due to insufficient reproducibility of LC-MS(/MS) mass spectra obtained with different instrument types.

Key words: Systematic toxicological analysis, General unknown screening, LC-MS, GC-MS, Mass spectral libraries

1. Introduction

The identification of drugs and toxic compounds, often at low levels, is an important goal in clinical and forensic toxicology, doping control analysis, and environmental analysis, where the compounds involved are often unknown. Gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography with diode-array detection (HPLC-DAD), and liquid chromatography-mass spectrometry (LC-MS) are the tools most often used in toxicology

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laboratories for identification, or confirmation of identity, of xenobiotics and their metabolites.

In all these fields, numerous methods have been developed for the analysis of particular target compounds, classes of compounds (e.g., therapeutic drugs, drugs of abuse, pesticides, environmental contaminants, and metabolites thereof), or for a more comprehensive screening of xenobiotics and their metabolites in biological samples. In fact, the screening and identification of compounds of interest before quantification is part of daily routine work (1, 2). Drug screening is a term that encompasses all the techniques allowing the detection in one run of a large range of compounds of pharmacological or toxicological interest in urine, plasma, serum, whole blood, and other body fluids, as well as hair or postmortem tissues or organs (3).

Many targeted screening methods involving single-stage mass spectrometry in the single ion monitoring (SIM) mode or tandem mass spectrometry in the selected reaction monitoring (SRM) mode have been developed for virtually all classes of drugs and toxic compounds. In addition to the selective and, if correctly applied (4), specific detection of the compounds targeted, they allow for their quantification.

The general unknown screening (GUS) of drugs and toxic compounds involves untargeted analytical techniques. Its aim is to detect as many compounds as possible in a matrix and tentatively identify them, either by comparison with libraries of mass spectra or by direct interpretation of an individual spectrum. Systematic toxicological analysis (STA) occupies an intermediate position between targeted and untargeted analysis. A limited (although sometimes very large) list of target compounds is screened for and, for those tentatively detected, rich and/or accurate mass spectral information is obtained, ensuring their specific identification.

This chapter focuses on recent STA and GUS procedures involving mass spectrometry and discusses the respective merits of GC-MS, LC-MS(/MS), and new sample preparation techniques.

2. GC-MS for STA and GUS: Recent Improvements

GC-MS has been the technique most employed for the GUS of compounds of toxicological relevance for the last three decades, owing to its universal fragmentation conditions and to the availability of huge mass spectral databases. In addition to being at the forefront of the development of GC-MS in clinical toxicology, in particular regarding GUS, the group of Maurer recently tested the freeware deconvolution software AMDIS (Automated Mass Spectral Deconvolution and Identification System) with their GC-MS GUS technique in urine (5). For this, after optimization of the AMDIS deconvolution and identification settings, they

compared the results obtained from 111 urine samples by manual and AMDIS data interpretation. They concluded that AMDIS gave results comparable or even superior to manual evaluation by an experienced toxicologist, but that it could only identify targets present in the user-defined MS library. As AMDIS-readable libraries have to be generated by users by converting commercial or personal libraries, this may narrow the range of toxicologically relevant compounds identified and is a current limitation of this promising tool.

Steiner and Larson (6) employed Direct Analysis in Real Time (DART), a new atmospheric pressure ionization technique that can be used for the analysis of solids, liquids, and gases with little or no sample preparation, merely by placing the test material into a heated gas flowing through the sampling area. Ionization in the positive mode is obtained by charging a heated helium gas stream, which subsequently reacts with the molecules on the surface of the sample to induce ionization. In this study, DART was coupled with a time-of-flight (TOF) MS analyzer operating at different collision-induced dissociation (CID) voltages, without prior chromatographic separation. This technique was able to detect many more compounds than GC-MS in 553 forensic case specimens; however, the authors emphasized that data obtained need to be examined very carefully as the spectra produced from multicomponent mixtures can become extremely difficult to interpret, interferences can result in falsely positive results, and differences in in-source CID spectra can arise for mixtures of compounds with widely varying proton affinities.

3. Recent LC-MS (/MS) Techniques for STA and GUS

Over the last 15 years or so, methods based on the use of HPLC coupled with single-stage or tandem MS detection have been reported for GUS and STA, fostered first by the necessity of detecting compounds not amenable to GC (i.e., highly polar, high-molecular-weight, or thermally labile compounds). It rapidly turned out that this coupling could detect a very large range of xenobiotics.

3.1. Single-Stage Quadrupole Mass Spectrometry

For single-stage MS, in-source CID at different energies was used to generate fragments and obtain rich enough spectra to be searched against libraries of spectra generated by the injection of reference materials in the same conditions. These methods, reviewed in detail elsewhere (7–9), have now been superseded by newer approaches.

3.2. Tandem Quadrupole Mass Spectrometry

Many LC-MS/MS methods have been published for the targeted analysis of a wide variety of drugs, mainly using SRM on triple quadrupole instruments. For instance, Gergov et al. (10) developed

a method for the screening of 238 drugs in blood using one SRM transition per compound and a compound-dependent collision energy (20, 35, or 50 eV), for a total cycle time of 6 s. However, the use of only one, or even two, SRM transition per compound is generally insufficient, yielding a significant number of false-positive findings (4). It should be kept in mind that MS in the SIM or SRM modes can never reach the identification power of a full mass spectrum (1).

Improvement with respect to these SRM methods was rendered possible by the availability of data-dependent acquisition or information-dependent acquisition (IDA), by which a tandem mass spectrometer can automatically switch from a "survey" mode to a "dependent" (or confirmation), full-spectrum MS/MS mode. In addition, the introduction of linear ion-trap-triple quadrupole (LIT-QqQ) hybrid instruments further extended the possibilities of LC-MS/MS in STA or GUS. In this instrument, the second mass analyzer can be used as either a conventional quadrupole mass analyzer or a linear ion trap, which by accumulation of ions provides enhanced full-spectrum sensitivity compared to a conventional quadrupole. The group of Weinmann used targeted SRM with up to 700 transitions as the survey detection mode, and the "enhanced" product ion (EPI) spectrum mode as the dependent mode (11). Whereas this procedure seems to be a more specific approach to STA as it allows searching rich spectra against those entered in libraries, the use of SRM as the survey mode cannot answer the more general clinical question as to whether an individual has been intoxicated at all, rather than intoxicated with a compound from a predefined list (12). Also, the use of only the positive-ion mode narrows the detection window.

Alternatively, the single-quadrupole, enhanced full-spectrum (EMS) mode has been used as the survey detection mode, with alternated polarities (13, 14). The major three ions in each Q3 MS were selected in the next three acquisitions and fragmented in the collision cell at three collision energies for each one, taking advantage of the accumulation capacity of the linear trap. Separate libraries were generated for the positive-ion and negative-ion modes by injecting pure solutions of drugs and toxic compounds, as well as by entering the MS/MS spectra of metabolites found in human samples, or even specifically produced by means of in vitro metabolic experiments (13). More than 1,000 MS/MS spectra in the positive mode and 250 in the negative mode were entered in the respective libraries, together with compound name, developed chemical structure, CAS number, retention time, relative retention time, and ultraviolet spectrum. A program was developed to automatically report the results of peak finding and library searching. Compounds not found by other screening or target techniques could be identified unambiguously by this LC-LIT-QqQ GUS technique in clinical toxicology cases (15). This technique is described in Chapter 11.

Libraries of mass spectra obtained through CID in the collision cell of triple quadrupole instruments have been developed for STA (16) or GUS (15). The robustness of CID mass spectra between instruments from the same or from different manufacturers, and thus the interchangeability of these libraries, has been investigated by different groups (17–22). These studies generally showed that the CID spectra were robust across laboratories equipped with the same instruments, or with instruments of the same brand, but that the relative intensity, and sometimes the nature of the fragments, differed across different instrument brands. However, in a recent study, product-ion spectra were generated at ten different collision energy values using a quadrupole-time-of-flight (Q-TOF) tandem mass spectrometer, filtered and entered in an MS/MS library. This library was further used to search unknown spectra generated on Q-TOF, QqQ, hybrid LIT-QqQ, and linear ion-trap-FTICR (Fourier transform ion cyclotron resonance) instruments in three different laboratories. By means of a sophisticated matching algorithm, the correct compound was retrieved as the best hit in 98.1% of cases and as the second best in the remaining 1.9% of cases (22).

Although also possible, the interpretation of unreferenced MS/MS spectra is a challenge because of the limited understanding of the fragmentation and rearrangement reactions involved and the limited number of fragments sometimes observed. As is seen below, even accurate-mass determination using high-resolution TOF or orbitrap mass spectrometers may not be sufficient to successfully identify unknowns.

3.3. Single-Stage Linear Ion-Trap Instruments

Mass spectral libraries dedicated to ion-trap instruments, whether three-dimensional or two-dimensional (i.e., quadrupole ion traps), have also been set up (23, 24), taking advantage of the easier-to-optimize CID conditions in ion traps due to the possibility of normalizing collision energies, and the more reproducible spectra obtained. Dulaurent et al. developed a GUS procedure for 320 pesticides and metabolites in blood using a linear ion-trap instrument in the positive and negative ions, MS^2 and then MS^3 modes (24). They generated MS^2 and MS^3 libraries of 450 and 430 spectra, respectively. Library searching was performed on MS^2 spectra and retention time, and positive results confirmed by manually checking the corresponding MS^3 spectrum. The limitations of this technique were that not all pesticides investigated could be detected and that the cycle time was quite long when continuously switching from the positive to the negative ionization modes. The authors admitted that, if necessary, it was possible to decrease the detection limits of some compounds by 10–100-fold by scanning MS^2 in only one polarity, owing to a shorter total scan time.

3.4. High-Resolution Mass Spectrometry

Liquid chromatography coupled to high-resolution TOFMS instruments, enabling accurate-mass determination, has also been employed for STA or GUS (25, 26). Identification has been based on the accurate mass, isotopic pattern, and retention time (27–29) of sample components, from which the atomic formula is calculated and searched against a database of relevant compounds, preferably using dedicated software (27). Alternatively, forward searching of compounds of toxicological interest in the full-scan TOFMS data was proposed by Ojanpera's group. This approach has been largely applied in the last couple of years in anti-doping laboratories (30–32). For instance, a generic LC-TOFMS method was developed and validated for 241 substances prohibited by the World Anti-Doping Agency, belonging to various categories (31). Positive identification was based on retention time and accurate mass, as compared to reference materials or compounds contained in urine samples from excretion studies. Limit of detection, extraction recovery, matrix effect, and repeatability were checked and the method successfully applied to the retrospective screening of a single designer drug, 4-methyl-2-hexanamine, in stored doping control samples.

When reference standards are not available, structures and thus elemental formulae of compounds of toxicological interest and their known or putative metabolites may be taken from the literature or inferred from expected metabolic pathways (33) and added to the database. However, as there are generally several compounds with the same elemental formula and molecular mass, and as their metabolites may also have the same masses, confirmation procedures may be necessary (1). Polettini et al. actually showed that no compound could be unambiguously identified in postmortem samples when searched against a library of 55,000 compounds of toxicological relevance (34). Lee et al. tried to overcome this limitation by using in-source CID to obtain more structural information and by building a mass spectral library using this approach (29). Alternatively to library searching, Pelander et al. relied on the prediction of fragmentation patterns using dedicated software (33). However, more application data will be necessary to demonstrate the reliability of compound identification without reference standards (2).

A next step in the development of LC-MS approaches in STA or GUS has been the use of two-stage, Q-TOFMS instruments able to generate accurate mass data of the parent as well as fragment ions directly attributable to the parent (21).

Only a few applications of orbital-trap (orbitrap) high-resolution mass spectrometers have been reported for STA or GUS so far. For the detection of 29 doping agents, an LTQ-orbitrap mass spectrometer equipped with an APCI ion source was used with in-source CID and acquisition in the positive ionization scan mode from 100 to 500 Da (35). The mass resolution of 60,000 full width at half maximum (FWHM) ensured a precision better than 2 ppm (using external calibration), while the limit of detection was better

than 100 pg/mL for all compounds. The possible fragmentation pathways of each agent were inferred from the fragments generated, using proprietor software. Despite the high selectivity of this technique, the authors admitted that some of the analytes were isomeric and had to be separated chromatographically. Using a different version of orbitrap, with no linear ion-trap upfront, Thomas et al. (36) developed a method without precursor ion selection, where spectra were acquired in the positive and negative modes in three alternated conditions: without fragmentation in the 100–1,000 Da range with a resolving power of 50,000 FWHM and then with CID at collision energies of 20 and 50 eV in the m/z 70–600 range with a resolving power of 25,000 FWHM. The resulting cycle time was <2 s. Compound identification was based on the accurate masses of the parent and fragment ions, sometimes both in the positive and negative ionization modes, as well as on their retention time. The authors validated their method for 32 doping agents, including some designer drugs recently introduced in the WADA lists for which no analytical technique was available at the time. Like the previous group, they emphasized the fact that this kind of method provides mass spectra containing all the desired information to identify unknown substances retrospectively.

A comparative study between TOFMS and orbitrap accurate mass spectrometry coupled with ultra-performance liquid chromatography (UPLC) was conducted in the field of hormone and veterinary drug residue analysis (37). Extracts from blank bovine hair were fortified with 14 steroid esters. All 14 compounds could be detected and their accurate mass measured at low ng/g concentrations using orbitrap mass spectrometry at a resolving power of 60,000. UPLC-orbitrap at a resolving power of 7,500 and UPLC-TOFMS at mass resolving power of 10,000 both failed to detect all steroid esters, owing to the inability to resolve analyte ions from co-eluting isobaric matrix compounds. High resolution can thus partly compensate for low signal-to-background noise concentration ratios, but the authors concluded that nonselective sample preparation is expected to aggravate the issue of false negative results obtained due to insufficient mass resolving power.

4. Ultrahigh Pressure (or Ultra-Performance) Liquid Chromatography

Quite a few of the recent, abovementioned STA or GUS techniques actually employed UPLC or UHPLC (29, 30, 36, 37) upfront mass spectrometry. However, the enhancement in chromatographic resolution produces very narrow (commonly 1–3 s wide) chromatographic peaks (38), which is only compatible with mass spectrometry cycle times at least threefold shorter (provided no polarity switching or alternated collision energies are used).

High-resolution mass spectrometers such as TOFMS or orbitrap instruments are more suited than QqQ instruments to acquire full-scan MS data within this time frame. High chromatographic resolution may thus be considered as a hindrance to rich MS data acquisition.

5. Comparison of GC-MS and LC-MS/MS Techniques for Screening Compounds of Toxicological Interest

Lee et al. compared their UPLC-TOFMS technique with HPLC-UV (REMEDiHS), in-house HPLC-DAD, full-scan GC-MS, and UPLC-MS/MS in the SRM mode for the analysis of 30 authentic urine samples (29). UPLC-TOFMS was able to detect 95 compounds, the REMEDiHS 47, GC-MS (without derivatization) 23, HPLC-DAD 14 (in a library of 594 UV spectra), and UPLC-MS/MS 23 (out of 170 targeted compounds). 94.7% of the compounds detected by TOFMS were confirmed by at least one of the other techniques, while the remaining four results could not be confirmed as false positive as the corresponding compounds were not included in the other techniques. On the other hand, three false negative results were noted. Although the "gold standard" comprised a combination of suboptimal techniques, these results advocate for the sensitivity and specificity of UPLC-TOFMS for GUS.

Lynch et al. compared five methods for GUS/STA (which they called comprehensive drug screening, or CDS) for their ability to detect drugs in 48 patient urine samples: LC-UV (REMEDi), full-scan GC-MS after acetylation of the extracts, full-scan LC-MS with in-source CID, LC-LIT-QqQ in the SRM information-dependent acquisition-enhanced product ion scan (SRM-IDA-EPI) mode (264 SRM transitions in the survey mode), and LC-LIT in the polarity switching, targeted MS^2 mode (39). They found that the LC-LIT and LC-LIT-QqQ methods identified 15% more drugs than the single-stage MS or LC-UV methods. However, none was able to detect all compounds and automatic library searching and reporting algorithms resulted in false positive and false negative results, which could be easily identified upon manual review of the raw data. The most common cause of false positive results was carryover, specially for LC-LIT, followed by nonspecific matching of spectra with <3 ions (in particular for LC-LIT-QqQ). It is worth noting that LC-LIT-QqQ led to tenfold more false negative results than LC-LIT (49.3% vs. 4.8%), which may also partly be attributed to the limited number of targeted SRM transitions with the former.

Another comparative study was conducted between GC-MS and an STA procedure developed on an LIT-QqQ instrument, following 100 drugs in the SRM survey mode (40). Ninety-five postmortem blood samples were analyzed in parallel resulting in

the detection of >400 drugs, and the two techniques yielded a surprising 98% concordance between them, despite 2 years of refrigerated storage between the two sessions of analyses.

One limitation of these three comparative studies is that the sample preparation procedures were different for all the analytical techniques compared, with or without urine hydrolysis, using liquid–liquid extraction (LLE), solid-phase extraction (SPE), or dilute and shoot, which does not actually allow for rigorous comparison of the respective merits of the hyphenated techniques. However, all three showed that LC-MS(/MS) was at least as efficient as the traditional techniques used for GUS/STA in most toxicology laboratories.

6. Extraction Strategies

Sample preparation and limits of detection are also important determinants of the efficiency of such methods. In particular, nonselective extraction procedures are necessary for good recovery of molecules in a wide polarity range, including highly polar drugs not amenable to GC-MS.

Filtration and injection or protein precipitation with acetonitrile and injection of the supernatant (the so-called dilute and shoot strategy) can provide a direct means to introduce samples into HPLC (41). However, the lack of a concentration step may limit the detection of some of the most potent drugs, while the absence of a purification step may favor matrix effects, hence false negative results.

Among the molecules targeted by LC-MS(/MS) GUS procedures are those not amenable to GC-MS, i.e., polar, acidic, thermally labile, or hydrophilic, and the extraction procedure should be chosen accordingly. Two LLE procedures can be used in parallel, one for acidic and one for basic compounds. SPE is also widely employed, either based on classical, mildly hydrophobic C8 or C18 mixed-mode phases, or on mixed-mode sorbents that can probably cope with compounds in the largest polarity range.

Though hardly addressed in the literature, emphasis should also probably be put on the very last step of sample preparation, i.e., the nature of the solvent used to reconstitute dry extracts, as the solubility of polar compounds can be poor in pure organic solvents.

Another recent possibility is to use online sample preparation techniques. Standard SPE cartridges can be used with commercial SPE automation coupled upfront with LC-MS/MS. Alternatively, microextraction by packed sorbent (MEPS) is a miniaturized SPE format intended to work with sample volumes as small as 10 µL. The MEPS sorbent bed is integrated into a syringe that allows for manipulations of low-volume samples, either manually or in

combination with certain autosamplers or sample preparation robots. The low solvent volume used for the elution of the analytes can be injected directly into GC or LC systems, hence providing completely automated MEPS/LC-MS or MEPS/GC-MS systems (42). Turbulent flow chromatography (TFC) is a column-switching technique based on direct injection of biologic samples, without previous extraction. Its main characteristic is the use of a first column packed with large particles of a stationary phase material and a high mobile phase flow rate, the combination of which generates a particular chromatographic behavior called turbulent flow, which allows retention of the small molecules of interest and exclusion of large biomolecules. However, drug–protein bonds have to be broken prior to injection into the system, generally using a first step of manual protein precipitation; otherwise the drug bound fraction would be eliminated with the proteins. To our knowledge, no published STA or GUS technique has employed online SPE, MEPS, or TFC so far. Although probably not superior to classic extraction techniques in terms of recovery yields and method sensitivity, these online techniques offer the advantage of automation. Actually, whatever the sample preparation procedure, one of the main problems when using LC-API-MS, particularly for GUS, is to detect even small signals against a high background noise. The critical point indeed is the signal-to-noise ratio (S/N), mainly determined by the purity of the extracts injected and the recovery of the compounds of interest.

7. Conclusion

Untargeted screening for unknown compounds by LC-MS is highly challenging. As a general rule, MS/MS in toxicology brings higher specificity and selectivity (higher S/N), as well as more structural information when an unknown chromatographic peak has to be explored. However, the first step for GUS is to detect unexpected compounds, which is not compatible with the classical SRM mode, either used alone or as the survey scan prior to a confirmatory, daughter ion scan mode. The major drawback of LC-MS/MS in the full-scan mode for STA or GUS is the lack of reference libraries that can be used on different apparatus types due to insufficient reproducibility of LC-MS(/MS) mass spectra obtained with different instrument types.

Major improvements have recently come from the MS part of the coupling: linear ion traps offer increased S/N ratio and MS^3 capabilities, while high-resolution (TOF or orbitrap) mass spectrometers offer higher mass precision, which greatly facilitates identification of unknown compounds and apparently shows the best performance in comparative studies. The time is probably

close now for a universal GUS procedure based on LC-MS, similar to but with much better performance than full-scan GC-MS, provided standardization of basic MS conditions can be agreed upon by vendors of mass spectrometers in order to share large libraries of spectra.

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