J Lab Med 2012;36(2):89–97 © 2012 by Walter de Gruyter • Berlin • Boston. DOI 10.1515/labmed-2011-0014

Drug Monitoring und Toxikologie/
Drug Monitoring and Toxicology

Redaktion: W. Steimer

Systematic toxicological analysis using liquid chromatography-mass spectrometry: techniques and inter-instrument reproducibility of mass spectra

Systematisch-toxikologische Analyse mittels Hochleistungsflüssigchromatographie-Massenspektrometrie: Techniken und Reproduzierbarkeit von Massenspektren zwischen Instrumenten

Frank T. Peters[1,*] and Dirk K. Wissenbach[2]

[1]Institut für Rechtsmedizin, Universitätsklinikum Jena, Jena, Germany
[2]Department Metabolomics, Helmholtz-Zentrum für Umweltforschung UFZ, Leipizg, Germany

Abstract

The so-called systematic toxicological analysis (STA) aiming at simultaneous analysis of as many toxicologically relevant compounds in biosamples as possible is an important part of routine analysis in clinical and forensic toxicology. Gas chromatography-mass spectrometry and liquid chromatography with diode array detection have been the most widely used techniques for this purpose. However, in recent years STA methods based on liquid chromatography coupled to mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) have become increasingly important, although their widespread use is still hampered by the lack of a universal reference library of mass spectra that can be used on all major instrument platforms. In this review, LC-MS(/MS) methods for STA in urine and/or blood published in the past 6 years will be compared and discussed with regard to sample preparation, separation, instrument types used for mass spectrometric detection, and method validation. In addition, different approaches to achieving the goal of a universal reference library will be summarized.

Keywords: accurate mass; library; liquid chromatography-mass spectrometry; mass spectra; systematic toxicological analysis.

*Correspondence: Priv.-Doz. Dr. rer. nat. Frank T. Peters, Institut für Rechtsmedizin, Universitätklinikum Jena, Fürstengraben 23, 07743 Jena, Germany
Tel.: +49-3641-9-35584
Fax: +49-3641-9-37902
E-Mail: frank.peters@med.uni-jena.de

Zusammenfassung

Die so genannte Systematisch-toxikologische Analyse (STA), sprich die simultane Analyse möglichst vieler toxikologisch relevanter Substanzen in biologischen Flüssigkeiten, ist ein wichtiger Bestandteil der Routineanalytik in der Klinischen und Forensischen Toxikologie. Bisher waren Gaschromatographie-Massenspektrometrie und Flüssigchromatographie mit Diodenarraydetektion die am weitesten verbreiteten Techniken für diesen Zweck. In den letzten Jahren haben jedoch Methoden an Bedeutung gewonnen, die auf der Kopplung von Flüssigchromatographie und Massenspektrometrie (LC-MS) oder Tandemmassenspektrometrie LC-MS/MS basieren, auch wenn ihre weitere Verbreitung durch das Fehlen einer universellen Massenspektrenbibliothek für unterschiedliche Geräteplattformen behindert wird. In der vorliegenden Übersichtsarbeit werden in den letzten sechs Jahren publizierte LC-MS(/MS)-basierte Methoden für die STA in Urin und/oder Blut verglichen und im Hinblick auf Probenvorbereitung, Trennung, verwendete Art von Massenspektrometern und Methodenvalidierung diskutiert. Darüber hinaus werden unterschiedliche Herangehensweisen bei der Entwicklung einer universellen Spektrenbibliothek zusammengefasst.

Schlüsselwörter: Bibliothek; Feinmasse; Flüssigchromatographie-Massenspektrometrie; Massenspektren; Systematisch-toxikologische Analyse.

Introduction

In clinical toxicology and in the closely related field of forensic toxicology, the so-called systematic toxicological analysis (STA) is an important part of daily routine work. It is an untargeted analytical approach ideally allowing sensitive detection and unambiguous identification of any toxicologically relevant compound in biosamples such as urine or blood. Although this ideal goal can only be

achieved with a combination of multiple analytical procedures, numerous methods covering at least hundreds of drugs, poisons, and/or their metabolites have been published and reviewed several times [1–6]. They are based on various chromatography-based techniques such as gas chromatography (GC) with different detectors, GC coupled to mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) with diode array detection (DAD), or HPLC coupled with single stage (LC-MS) or tandem (LC-MS/MS) mass spectrometry. In most forensic and clinical toxicology laboratories, GC-MS and HPLC-DAD are still primarily used for STA. GC-MS combines the very high separation power of GC with the high selectivity of electron ionization (EI) MS. However, it is limited to water-free sample extracts and volatile and thermostable analytes. HPLC-DAD has a high separation power and also allows analysis of aqueous samples and of rather hydrophilic, thermolabile, and less volatile analytes. However, its sensitivity is generally lower than that of GC-MS.

For these reasons, several research groups have worked on STA procedures combining the high selectivity of mass spectrometric detection with the possibility to directly analyze aqueous samples and hydrophilic, thermolabile, and non-volatile analytes. This resulted in the development of STA methods on different types of LC-MS and LC-MS/MS instruments which have become increasingly important in recent years. The widespread and effective use of these techniques has so far been hampered by 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. However, most recent developments on sophisticated algorithms for library searching may soon help to finally overcome this problem.

In this review article, LC-MS- and LC-MS/MS-based methods for STA in clinical and forensic toxicology covering at least several hundred analytes in blood, plasma, serum, or urine and published in the past 6 years will be reviewed and discussed with particular regard to the different mass spectrometric techniques employed. Moreover, the reproducibility of mass spectra between instruments and recent developments in the area of inter-instrument reference library transfer will be discussed.

Liquid chromatography-(tandem) mass spectrometry-based methods for systematic toxicological analysis

Analytical methods for STA in clinical and forensic toxicology have been developed for different types of mass spectrometric instruments such as: triple quadrupoles (QQQ) [7], hybrid triple quadrupole-linear ion trap (QQQ-LIT) [8, 9] instruments, ion trap (IT) [10] and linear ion trap (LIT) [11–14] instruments, time-of-flight (TOF) [15–19] instruments, and quadrupole-time of flight (Q-TOF) [20, 21] instruments. Key information of the reviewed STA methods is summarized in Table 1.

Biosamples for systematic toxicological analysis using liquid chromatographic-mass spectrometric techniques

A look at the sample matrices shows that with exception of one [7], all of the methods listed in Table 1 were either exclusively or additionally developed for analysis of urine, a well-established matrix for screening analysis. Urine samples are generally available at comparatively large volumes and can be obtained non-invasively. Moreover, drugs and/or their metabolites are more concentrated in urine [6]. If urine is not available, it can be necessary to perform STA in whole blood, plasma, or serum. However, these matrices are less suited for STA because the expected analyte concentrations are much lower. Whereas the paper published by Humbert et al. [7] only describes a work-up procedure for serum, other methods additionally cover further matrices [8, 9, 11, 20, 21]. Plasma was used in two other methods [9, 20, 21] and whole blood was only used in the methods primarily developed for forensic purposes such as postmortem toxicology [10, 17, 20, 21]. Sauvage et al. [9] also included analysis of gastric content in their method.

Sample preparation for systematic toxicological analysis using liquid chromatographic-mass spectrometric techniques

Methods for STA are supposed to cover analytes with a wide range of physicochemical properties. The sample preparation for such methods must therefore be unselective to ensure that relevant compounds are not eliminated during work-up. The least selective sample preparation techniques are dilution and/or protein precipitation because all compounds present in the original biosamples will also be contained in the prepared samples. This approach was used by several authors [8, 12, 13, 20, 21]. A drawback of dilution/protein precipitation is that the analyte concentration is diluted leading to lower sensitivity of the method unless the supernatant is reconcentrated by evaporation of the dilution/precipitation solvent as described by Wissenbach et al. [12, 13].

If samples are extracted by liquid-liquid extraction (LLE), it is important that this is done at an acidic as well as a basic extraction pH to extract acidic, neutral, and basic compounds. The two extracts can either be analyzed separately or pooled and analyzed in a single run as described by Humbert et al. [7] and Lee et al. [19]. In the experience of the authors of this review, LLE at only a pH around 9 as described by some authors [8, 10, 17] will also provide sufficient extraction yields for high-dosed acidic compounds such as many non-steroidal anti-inflammatory drugs, but has to be complemented by an acidic extraction for a truly comprehensive screening [22].

Offline and online solid-phase extraction (SPE) can be performed with different types of extraction sorbents. Reversed-phase sorbents such as the C18 material employed for online-SPE by Mueller et al. [14] can retain a wide spectrum of compounds with lipophilic properties, whereas hydrophilic compounds maybe poorly retained or not retained at all. A slightly more polar polymer-based polydivinylbenzene

Table 1 Key information on liquid chromatography-(tandem) mass spectrometry-based methods for systematic toxicological analysis.

Instrument type	Analytes	Sample matrix	Sample preparation	Stationary phase	Mobile phase and run time	Ionization and detection mode	References
QQQ	500 Toxicologically relevant analytes	S	LLE (pH 3.5 and 9–9.5)	Acquity UPLC HSS C18 150×2.1 mm, 1.8 µm	15 min gradient: 5 mM aq AF buffer and ACN with 0.1% FA	ESI+ and ESI–, FS, in-source (CID) fragmentation at 6 voltages per polarity	[7]
QQQ-LIT (QQQ)	700 Drugs, pharmaceuticals and toxic compounds	U S	PP/dilution, LLE (pH 9) LLE (pH 9)	Allure PFP Propyl 50×2.1 mm, 5 µm	17.5 min gradient: water and ACN with 2 mM AF and 2% FA each	ESI+MRM (survey), DDA, EPI with CES	[8]
QQQ-LIT	>1000 Drugs, toxic compounds and some metabolites	S, P, U, G	SPE (HLB)	XTerra MS C18 100×2.1 mm, 3.5 µm	25.5 min gradient: 0.5 mM aq AF and ACN with 10% 10 mM aq AF	ESI+ and ESI–EMS (survey), DDA, EPI with CES	[9]
IT	>800 Toxicologically relevant compounds	B, U B, U H	SPE (HCX) basic fraction LLE (pH 9) Sonication with 0.1 M HCl, LLE (pH 9)	Zorbax SB-Aq 100×2.1 mm, 1.8 µm	15 min gradient: 0.1% aq FA and MeOH	ESI+, FS (survey), DDA, PIS with CE ramping	[10]
LIT	365 Drugs and important metabolites	S, U	PP online SPE (DVB)	Nucleodur C18 Gravity 100×3 mm, 3 µm	23 min gradient: 10 mM AF and ACN	APCI+ and APCI–, FS (survey), DDA, PIS	[11]
LIT	>900 Drugs and toxic compounds+2300 metabolites	U	PP	Hypersil GOLD C18 100×2.1 mm, 1.9 µm	25 min gradient: 10 mM aq AF with 0.1% FA and ACN with 0.1% FA	ESI+, FS (survey), DDA, PIS (MS^2 and MS^3)	[12, 13]
LIT	356 Drugs and some metabolites	U	(EnHy) online SPE (C18)	Betasil Phenyl/Hexyl 100×3 mm, 3 µm	31.8 min gradient: 5 mM aq AA with 0.1% FA, 5 mM AA in MeOH with 0.5% FA, and iPrOH/acetone/ACN	APCI+ and APCI–, FS (survey), DDA, PIS (MS^2 and MS^3)	[14]
TOF	735 Drug and metabolites (7640 target compounds)	U	EnHy, SPE (HCX) acidic/ neutral and basic fraction	Luna-C18(2) 100×2 mm, 3 µm	27 min gradient: 5 mM aq AA and ACN	ESI+, FS	[15, 16]
TOF	Approx. 50,500 compounds	H B U	Incubation with 0.1 HCl, LLE (Toxitube A) PP, LLE (pH 8.9) Dilution	Zorbax Eclipse C18 150×2.1 mm, 3.5 µm	40 min gradient: 0.1% aq FA and ACN	ESI+, FS	[17, 18]
TOF	Over 300 common drugs and metabolites	U	LLE (pH 3.5 and 9–9.5)	Acquity UPLC HSS T_3 100×2.1 mm, 1.8 µm	14 min gradient: 0.05% FA and MeOH	ESI+, FS, in-source (CID) fragmentation at two aperture voltages	[19]
QTOF	2500 Toxic compounds	B, S, P U	PP and LLE (acidic pH and pH 9) Dilution	Poroshell 120 EC-C18 100×2.1 mm, 2.7 µm	27 min gradient: 10 mM aq AA and MeOH	ESI+, FS (survey), DDA, PIS with *m/z*-dependent CE	[20, 21]

QQQ, triple quadrupole; QQQ-LIT, hybrid triple quadrupole with linear ion trap option; IT, ion trap; LIT, linear ion trap; TOF, time-of-flight; Q-TOF, quadrupole-time-of-flight; S, serum; U, urine; B, blood; P, plasma; G, gastric content; H, hair; LLE, liquid-liquid extraction; SPE, solid-phase extraction; HCX, mixed-mode sorbent with hydrophobic and cation exchange properties; PP, protein precipitation; EnHy, enzymatic hydrolysis; aq, aqueous; AF, ammonium formate; ACN, acetonitrile; FA, formic acid; MeOH, methanol; AA, ammonium acetate; iPrOH, iso-propanol; ESI, electrospray ionization; FS, full scan; CID, collision-induced dissociation; MRM, multiple-reaction monitoring; DDA, data-dependent acquisition; EPI, enhanced product ion; EMS, enhanced mass spectrometry; CES, collision energy spread; PIS, product ion scan; APCI, atmospheric pressure chemical ionization.

(DVB) sorbent was used in the online-SPE method of Sturm et al. [11], whereas Sauvage et al. [9] used a polymer-based HLB (hydrophilic-lipophilic balance) sorbent with more pronounced polar character providing more interaction sites for polar compounds and making the sorbent easily wettable. With all three sorbents high extraction recoveries were observed for compounds from different drug classes. Ojanpera et al. [15, 16] employed a so-called mixed-mode SPE with an extraction cartridge containing a reversed-phase as well as a cation-exchange sorbent (HCX). With such cartridges the acidic/neutral analyte fraction can be extracted via the reversed-phase part of the sorbent, whereas basic analytes can be effectively extracted via the cation-exchange part. In the described method [15, 16], the fractions were eluted separately but then combined for simultaneous analysis of both fractions as discussed above for LLE. Using only the basic elution fraction of SPE on HCX cartridges as described by Liu et al. [10] results in a very clean extract, but is limited to basic compounds and hence less appropriate for STA.

Considering that almost all of the described methods were also used for urine samples, it is remarkable that only two [14–16] employed cleavage of glucuronic or sulfuric acid conjugates (phase II metabolites), although it is well-known that such conjugates are poorly extracted by most extraction methods. An alternative to conjugate cleavage is to use a very unselective sample preparation such as dilution and/or protein precipitation and to include the phase II metabolites in the reference library as described in the references [12, 13, 17]. In this case, however, it must be considered that all compounds being only partly conjugated will elute in two smaller peaks (one for the conjugate and one for the unconjugated compound) rather than one abundant peak of the unconjugated compound after previous hydrolysis and hence be less sensitively detected.

Chromatographic separation and ionization in systematic toxicological analysis using liquid chromatographic-mass spectrometric techniques

A common feature of all methods listed in Table 1 is the use of reversed-phase stationary phases and mobile phase gradients. With the exception of the method described by Mueller et al. [14] (ternary gradient), all procedures employed binary mobile phase gradients consisting of an aqueous part, acetonitrile [7–9, 11–13, 15–17] or methanol [10, 20, 21] as organic modifiers, and formic acid [7, 8, 10, 12–14, 17, 19], and/or the volatile buffers ammonium formate [7–9, 11–13] or ammonium acetate [14–16, 20, 21]. Such separation systems are very common in LC-MS(/MS) and will therefore not be further discussed here. However, it is worth mentioning that the run times (including re-equilibration before the next injection) of all methods ranged from 14 to 40 min (Table 1). The reasons are clearly that especially in the data-dependent acquisition (DDA)-based detection modes the risk of overlooking a relevant compound increases with the number of co-eluting analytes and decreasing width of the analyte peaks. In fact, even research groups using separation columns with sub-2 μm particle packings [7, 10, 12, 13, 19] did not fully exploit the high separation power of such stationary phases to ensure that the analyte peaks were wide enough to obtain at least two measurements per peak even when switching between different in-source collision-induced dissociation (CID) settings [7, 19] or going through DDA-MS^2-MS^3 cycles [12, 13].

Most of the methods reviewed here used electrospray ionization (ESI). However, Sturm et al. [11] and Mueller et al. [14] used atmospheric pressure chemical ionization (APCI), because this ionization mode is well-known to be less prone to matrix effects, i.e., ion suppression or enhancement [23]. Indeed, neither Sturm et al. [11] nor Mueller et al. [14] observed any major matrix effects. However, this may not only be attributable to the ionization mode but also to online extraction prior to analysis. Of those groups having checked for matrix effects in the ESI mode, Sauvage et al. [9] and Lee et al. [19] who had used more extensive sample clean-up by SPE and LLE, respectively, either reported no matrix effects at all [9] or only for few analytes [19]. In contrast, Wissenbach et al. [12, 13] who had used simple dilution/protein precipitation observed, in part, extensive but still acceptable ion suppression or enhancement effects.

Considering that one of the primary goals of STA is to cover as many analytes as possible within a single analytical procedure and that toxicologically relevant compounds with acidic properties are generally more readily ionized in the negative mode, one would expect that LC-MS-based methods for STA would switch between positive and negative ionization within the same analytical run. However, positive and negative ionization was only reported for four of the reviewed methods [7, 9, 11, 14]. Sturm et al. injected samples twice, once in the positive and once in the negative mode, which on the one hand expands the spectrum of analytes covered, but on the other hand is time-consuming [11]. Humbert et al. [7] stated for their method that when scanning only in the positive mode "these settings resulted typically in a minimum of 10 data points per peak", suggesting switching of polarities was not routinely used by these authors. Sauvage et al. [9] reported settings for both positive and negative mode but did not specify if switching between polarities was used. However, Dresen et al. [8] who used the same type of instrument stated that polarity switching was too slow for combining positive and negative ionization within the same run. Hence, the only method explicitly employing within-run polarity switching is the one described by Mueller et al. [14]. Nevertheless, the analyte spectrum of the latter method is limited by the fact that only compounds on a target list can trigger MS^n experiments to obtain product ion spectra (PIS) for library searching (see below).

Triple-quadrupole-based instruments

All instruments used in STA methods published after 2005 that have used quadrupole mass filters were QQQ or QQQ-LIT instruments. Dresen et al. [8] and Sauvage et al. [9] have described STA methods on QQQ-LIT that follow a similar general approach. Both methods are based on a survey scan,

DDA, and enhanced product ion (EPI) scanning followed by a library search.

In the method published by Dresen et al., the survey scan is performed in the multiple-reaction monitoring (MRM) mode, monitoring one transition for each of the 700 compounds included in the method. If one of these transitions reaches an abundance of 1000 counts per second or more, DDA triggers the EPI mode. Although this approach allows sensitive detection of the targeted compounds, it is inherently limited to these analytes. In the method described by Sauvage et al., the survey scan is performed in the so-called enhanced MS (EMS) mode, a single stage full scan mode in which ions are accumulated using the LIT function of Q3 before going to the detector to increase sensitivity. No intensity threshold was used in this method, thus any signal could trigger the EPI mode.

To avoid large and broad peaks exclusively and repeatedly triggering the MS/MS, the two most intense signals above the threshold [8, 9] or three most intense ions [24] could trigger the EPI mode. In addition, both groups used the feature of dynamic exclusion, i.e., survey signals having triggered the EPI mode twice [8] or four times [9] were excluded from DDA for 15 s. In addition to that, Sauvage et al. used dynamic background subtraction, in which the previous EMS scan is subtracted from the next, so that only rising peaks will have a positive abundance and can trigger the EPI mode [9]. Although these features can certainly limit the risk to overlook minor peaks, this could nevertheless happen, if numerous peaks co-elute, especially when using the EMS survey scan in which abundant peaks of entirely irrelevant compounds may still trigger the EPI mode.

When recording EPI spectra, both groups used the so-called collision energy spread (CES) feature by which fragment ions obtained at three different collision energies are accumulated by the LIT function of Q3 and scanned in a single, generally fragment- and thus information-rich EPI spectrum that is suitable for library searching. However, the CES setting used differed with Dresen et al. using a lower average energy and a smaller spread (35±15 eV) in comparison to Sauvage et al. (40±25 eV). Another difference is that the latter also recorded EPI spectra in the negative mode at –40±25 eV.

Despite using a QQQ instrument the MS strategy used in the STA method described by Humbert et al. [7] is closer to the single quadrupole methods published before triple quadrupoles became widely available. The QQQ instrument was not operated in any of the common MS/MS modes but rather in the simple full scan mode. Hence, fragmentation was not performed in Q2 as in most QQQ methods but rather in the form of in-source CID. Different fragmentation energies were achieved by varying the cone voltage of the instrument. CID spectra were recorded in both positive and negative ESI mode and at six different cone voltages for each polarity. At least one of these spectra was generally fragment- and information-rich and therefore useful for library searching. Nevertheless, the authors' statement that "in contrast to classical MS-MS which typically involves monitoring of the protonated molecular species in combination with just one or two product ions, the full scan approach can produce a wealth of identification point for each analyte" seems somewhat beside the point. It not only ignores that methods based on product ion scanning, such as those discussed above, also provide information-rich spectra but also that in such methods the origin of the fragments is known which increases the certainty of identification.

Ion trap and linear ion trap instruments

All four STA methods based on (linear) ion trap technology were based on library searching of PIS recorded in MS^2 mode only [11, 25] or in MS^2 and MS^3 mode [12–14]. All four methods used MS^1 in the full scan mode as the survey scan and DDA for triggering MS^2 and MS^3 experiments. However, the criteria for triggering such experiments were different. Mueller et al. [14] used a predefined list of precursor ions that could trigger MS^n experiments when reaching a certain intensity threshold. Hence, this method will only identify compounds with precursor ions included in that particular list, leading to target analysis rather than a general screening. The other authors also used intensity thresholds for DDA, but the most abundant [11], the four most abundant [12, 13], or the five most abundant ions [10] of the MS^1 would trigger MS^n experiments followed by dynamic exclusion after one [11] or two [10, 12, 13] occurrences. Energy ramping [10] or normalized collision energies [11–14] were used to generate fragment-rich PIS suitable for library searching. To further enhance the mass spectral information for compound identification, Wissenbach et al. [12, 13] and Mueller et al. [14] further recorded MS^3 spectra that were included in the library search.

Time-of-flight and quadrupole-time-of-flight instruments

STA using TOF-based mass spectrometers is based on high-resolution mass spectrometry (HRMS) alone or in combination with fragment-rich mass spectra. The Ojanpera group was the first to apply the concept of high-resolution mass spectrometry to STA in forensic toxicology [15, 16, 26–28]. It is based on the fact that with the high mass spectrometric resolving power of TOF instruments the molecular mass and the isotopic pattern of a compound can be measured with sufficient mass accuracy (in the low ppm range) to narrow down its elemental composition to very few or even a single elemental formula. In the latter case, the compound in question can be differentiated from isobaric compounds, i.e., compounds with the same nominal mass, but different elemental composition. An advantage of this approach is that reference libraries can be easily created by listing the accurate masses of relevant compounds. Thus, in addition to an in-house database of 735 analytes for routine analysis, Ojanpera et al. generated a large database with 7640 entries by calculating the accurate masses of compounds included in a large GC-MS reference library. Polettini et al. [18] generated an even larger accurate mass library of over 50,000 pharmacologically or toxicologically relevant compounds by downloading the respective accurate

mass data from the internet. However, an important disadvantage of compound identification by HRMS and accurate mass alone is that isomers, i.e., compounds with the same elemental composition such as the opioid analgesic tramadol and the antidepressant metabolite *O*-desmethylvenlafaxine, cannot be differentiated by this approach [16]. For this reason, unambiguous identification of isomers requires additional information such as retention times or fragmentation patterns. It was attempted to enhance the certainty of identification by predicting metabolites using in silico methods and including their accurate masses in the reference libraries and searches [29] or to shorten the hit list by systematically checking the HRMS data files for ions which would be in line with the presence of metabolites formed by major metabolic reactions [17]. Although none of these methods has fully solved the problems of isomers in HRMS-based STA, the approach is a powerful and efficient screening tool if combined with appropriate confirmation of the results.

Lee et al. [19] addressed this problem by including chromatographic retention data into the search strategy and by recording spectra at two aperture voltages, one leaving the intact pseudomolecular ion, one leading to in-source CID and more fragment-rich spectra increasing the confidence in identification considerably. However, as already mentioned above, using two aperture voltages increased the MS cycle time of the method necessitating a slower chromatography and hence partly compromising the major advantage of the used stationary phase.

The STA method by Broecker et al. [20, 21] can be seen as a combination of HRMS and fragment-rich mass spectra. Using a QTOF instrument, this group employed DDA as described above for the QQQ(-LIT), IT, and LIT methods. The survey scan was a high-resolution full scan and the three most abundant ions of each scan that reached a threshold abundance of 1000 counts/s triggered an MS/MS experiment. In this experiment, the precursor ion was isolated at low resolution and fragmented in a collision cell with mass-dependent collision energy. The resulting fragment-rich PIS were recorded in high resolution adding more information for identification via library searching. Although the reference library used in these experiments contained spectra recorded at fixed collision energies, generally good agreement of the mass spectra obtained with mass-dependent collision energy and the reference spectra recorded at the closest collision energy was observed. It can be expected that such methods combining the identification power of HRMS and fragment-rich spectra will become increasingly important in the future.

Method validation

Qualitative LC-MS(/MS)-based methods such as the STA methods reviewed here should at least be evaluated with regard to selectivity, limits of detection (LOD), and matrix effects [30]. However, with hundreds of compounds being covered by these methods, it is not possible to perform validation experiments for every single analyte. Most authors have therefore performed validation experiments for a more or less extensive subset of analytes [7–14, 19–21] to evaluate selectivity/specificity [14, 19], sensitivity [7, 8, 10–14, 19, 20], recovery [9, 12–14, 19], matrix effects [9, 12–14, 19], process efficiency (combination of recover and matrix effects) [11–13], carryover [14], and sample stability [31]. Most of them also performed systematic studies to compare their new LC-MS(/MS)-based screening methods with STA methods established in their laboratories to demonstrate the applicability of the new methods for the intended purpose [8–11, 14, 19, 32]. Further parameters evaluated by some authors were the reproducibility of retention times [7, 11, 14] and mass spectra [7, 13, 14]. Scientists looking for guidance on how to validate a LC-MS(/MS)-based STA method may use the paper published by Mueller et al. [14] as a template for their validation experiments.

Inter-instrument and cross-platform transfer of LC-MS/MS spectra

Depending on the type of mass spectrometer used, CID spectra can be generated in-source, "tandem in space" (QQQ) or "tandem in time" (IT or LIT) fragmentation [33]. Reproducible LC-MS/MS spectra can be obtained on specific instruments or instrument types from the same manufacturer and commercial reference libraries, and software packages are available from the manufacturers for certain instruments using the same fragmentation type. However, depending on kinetic and instrument parameters CID fragmentation patterns may differ considerably between different instrument types. For these reasons, no universal LC-MS/MS library has been established so far.

Two main strategies have been followed to achieve the transfer of MS/MS spectra from one LC-MS/MS fragmentation type to the other (cross-platform transfer), as well as from the same fragmentation type on different MS devices (inter-instrument). The first strategy is to achieve similar fragmentation patterns on different instruments by standardization of the fragmentation parameters using tuning compounds/protocols [34–38]. In this approach, the MS/MS settings of one instrument are systematically changed until a fragmentation pattern comparable to a reference spectrum recorded on a different type of instrument is achieved. Using standardized fragmentation parameters highly reproducible MS/MS spectra were obtained by different research groups, which can at least be successfully applied to a single instrument type from the same and different manufacturers [39–42]. Jansen et al. [43] found a rather poor MS/MS spectra reproducibility and significant differences for the relative fragment intensities even when using standardized collision parameters on four different QQQ(-LIT) instruments. As a result, these authors suggested that library search algorithms for LC-MS/MS spectra should put more weight on the presence of fragments rather than their relative abundance. The second approach to achieve an inter-instrument and cross-platform transfer of MS/MS spectra is collecting product ion spectra at several different collision energies [9, 44–50], so that the library will reflect various fragmentation conditions which

should account for fragmentation differences between instruments. It was shown that these strategies alone or in combination can result in good inter-instrument transferability as shown by Gergov et al., who investigated different QQQ LC/MS/MS spectra [46]. Considering all these systematic studies and their largely promising results, it is remarkable that a robust and universal LC-MS/MS library is still not available so far.

Other groups [51–53] have therefore tried to overcome this problem by sophisticated search algorithms putting less or no weight on absolute/relative abundance of fragments as suggested by Jansen et al. [43]. Oberacher et al. [51, 52] compared 418 MS/MS spectra of 22 compounds based on a reference library created by Pavlic et al. [47] using "tandem in space" and "tandem in time" instruments and such a sophisticated library search algorithm MS for ID (www.msforid.com) in a comprehensive multicenter study design. They found that a reference library containing QQQ MS/MS spectra recorded at different collision energies could be successfully used on other QQQ or (L)IT instruments from the same or different manufacturers, if the MS for ID search algorithm was used. With this approach, over 98% of the conducted library searches were correct, even if the author had eliminated some recorded spectra because of detection problems. To confirm the applicability of the MS for ID search algorithm and to evaluate the performance of two commercially available LC-MS/MS libraries, Oberacher et al. [54] compared the Pavlic et al. [47] and the Dresen et al. [49] reference libraries and could show that this search algorithm in principle provides good screening results with both reference libraries. Additionally, using the data from a previous study [51, 52] it was found that the Pavlic et al. [47] reference library showed a better "cross-platform" transferability in comparison to the Dresen et al. [49] library. The authors explained this by the greater number of different reference spectra for each compound stored in the Pavlic et al. library [47]. Nevertheless, the observed performance of this reference library [47] could be further improved by limiting the number of fragments in each spectrum to 16.

A further approach to library transfer was described by Wissenbach et al. [55]. As a first step, these authors modified their reference library recorded on a LIT instrument [12, 13] by adding spectra with an artificially enhanced abundance of the pseudomolecular ion and by merging the MS^2 and MS^3 reference spectra. After standardization of fragmentation, this library was then used in combination with a sophisticated search algorithm [53] on a QQQ-LIT instrument. Comparing the screening results of 100 urine samples obtained with the LIT and the QQQ-LIT instrument a 90% agreement was observed.

Conclusions and perspectives

In recent years, a large number of powerful LC-MS(/MS)-based procedures have been published for STA in clinical and forensic toxicology. Most of these methods use the classical STA matrices urine and blood and more or less sophisticated sample preparation. Run times of at least approximately 15 min are required to achieve sufficient separation of hundreds of analytes and to provide sufficient time for sophisticated DDA-based MS/MS methods. All major types of MS instruments commonly used in clinical and/or forensic toxicology laboratories have been employed in these methods and compound identification in these methods was either based on fragment-rich mass spectra followed by library searching, on accurate mass and HRMS, or combination of both. Most of the methods have been validated with regard to selectivity, sensitivity, and matrix effects and compared to established screening procedures by other techniques such as GC-MS and HPLC-DAD. In addition to the development of these STA methods, considerable progress has been made towards a universal mass spectral library that can be used on different instruments. Altogether, this will most probably increase the importance of LC-MS(/MS)-based methods in STA.

Acknowledgments

We thank Daniela Remane and Sven Baumann for their assistance.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.

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