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Review

Liquid Chromatography-Tandem Mass Spectrometry – Application in the Clinical Laboratory

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This review provides a concise survey of liquid chromatography-tandem mass spectrometry (LC-TMS) as an emerging technology in clinical chemistry. The combination of two mass spectrometers with an interposed collision cell characterizes LC-TMS as an analytical technology on its own and not just as a more specific detector for HPLC compared with conventional techniques. In LC-TMS, liquid chromatography is rather used for sample preparation but not for complete resolution of compounds of interest. The instrument technology of LC-TMS is complex and comparatively expensive; however, in routine use, methods are far more rugged compared to conventional chromatographic techniques and enable high-throughput analyses with very limited manual handling steps. Moreover, compared to both gas chromatography-mass spectrometry (GC-MS) and conventional HPLC techniques, LC-TMS is substantially more versatile with respect to the spectrum of analyzable compounds. For these reasons it is likely that LC-TMS will gain far more widespread use in the clinical laboratory than HPLC and GC-MS ever did. In this article, the key features of LC-TMS are described, method development is explained, typical fields of application are discussed, and personal experiences are related. Clin Chem Lab Med 2003; 41(2):117–126

Key words: Liquid chromatography-tandem mass spectrometry; Application; Clinical laboratory.

Abbreviations: APCI, atmospheric pressure chemical ionization; CID, collision-induced disintegration; ESI, electrospray ionization; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MRM, multiple reaction monitoring; LC-MS, liquid chromatography mass spectrometry; LC-TMS, liquid chromatography-tandem mass spectrometry.

Introduction

While chromatography and mass spectrometry represent key technologies in biomedical research, their application in the setting of the clinical laboratory has remained limited and restricted to rather few specialized laboratories. This is explained by the fact that these methods require – in contrast to contemporary clincical chemistry and immunoassay analyzers – very skillful handling and often cumbersome troubleshooting.

Typical applications of high-performance liquid chromatography (HPLC) in clinical chemistry are, for example, the quantification of urinary catecholamines and metabolites (metanephrines, vanillin mandelic acid, homovanillinic acid), of urinary 5-hydroxyindole acetic acid and serotonin, porphyrins, the measurement of serum vitamin A, E, B_1, B_6 concentrations, and the monitoring of some drugs (*e.g.*, amiodarone and itraconazole). Other HPLC methods compete with commercially available automated immunoassays (*e.g.*, 25-OH-vitamin D; homocysteine, desoxypyridinolin). Typical clinical applications of gas chromatography-mass spectrometry (GC-MS) are the quantification of organic acids and amino acids, profiling of fatty acids, and the detection of drugs of abuse and toxic compounds in forensic and environmental medicine, in addition to the search for inborn errors of metabolism.

Methodology of GC-MS

In GC-MS, the volatile eluent from the several meters long GC-capillary column is introduced directly into a high-vacuum area; here the compounds are ionized and disintegrated by the impact of accelerated electrons generated by a heated filament. This "electron impact" (EI-) ionization is disintegrating; the intact molecular ion is usually not generated. The fragments are then "filtered" according to their specific mass-to-charge ratio (m/z), as most widely used by a system of four parallel metal bars under a radiofrequency electromagnetic field (quadrupole) or a strong magnetic field. At a specific pattern of the radiofrequency field only molecules of one defined m/z ratio can pass the quadrupole and reach the detector, where electrons are liberated from an aluminum block. These secondary electrons generate a light signal on a membrane of phosphorus that is multiplied and quantified. Within a second or less the passing m/z ratios can be scanned over a range of 0 to about 800. Using this scan mode a disintegration spectrum of a compound eluted at a certain time point from the GC can be acquired; usually several typical fragment ions of an individual compound can be found reproducibly in the manner of a "fingerprint". This scan mode enables the identification of unknown compounds in biological materials by comparison with libraries of disintegration spectra. For

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quantification applications, however, usually the quadrupole alternates just between one typical fragment ion of the analyte and one fragment ion of an added internal standard compound to pass (selected ion recording, SIR). GC-MS has been in use in specialized clinical laboratories since the early '70s and enables highly specific and sensitive quantification of a limited number of certain medically important volatile compounds; polar compounds have to be derivatized prior to GC to offer appropriate volatility.

Methodology of Liquid Chromatography Mass Spectrometry (LC-MS)

With the development of HPLC technologies in the late '70s and early '80s the application of mass spectrometry as a detection principle for this more versatile chromatographic technique became tempting. However, the technical problems of LC-MS coupling are enormous. In GC-MS, the ionization of the volatile chromatographic eluent is performed within the high-vacuum region of the instrument; given typical GC-flow rates of 1 ml of helium per minute, turbomolecular vacuum pumps are capable of maintaining adequate vacuum within the instrument to allow ions to pass relevant distances without collision with air constituents. A direct flow of the liquid eluent of HPLC into, and evaporation of, the solvent within a mass spectrometer would produce enormous volumes of gas and a vacuum could never be maintained. Therefore, it was necessary to achieve evaporation of the solvents and ionization of the analytes outside the high-vacuum region at atmospheric pressure to make mass spectrometry applicable to HPLC.

Atmospheric pressure ionization

In this key feature of LC-MS technology, most commonly a 10%-split fraction of the HPLC eluent flow is sprayed through a capillary assisted by a flow of pressurized nitrogen to form an aerosol. The capillary bears a charge of 2.0–3.5 kV and consequently strong electrical charge is transferred to the droplets of this spray ("electrospray"). The aerosol is dried by a high flow of nitrogen (about 70 l/min) at a temperature of 200–300 °C; thus the HPLC solvent is evaporated from the droplets, they undergo a reduction in size, and the surface charge increases rapidly leading to the so-called Coulomb reaction. This produces new generations of smaller droplets until they have attained a sufficient charge density to allow sample ions to be ejected from the surface of the droplets ("ion evaporation"; Figure 1). In this way ionization is achieved within a distance of few millimeters from the tip of the capillary. The principle of electrospray ionization has been honored by the Nobel Prize in Chemistry 2002. Despite the high temperatures of nitrogen involved in this process, molecules do not experience thermal damage since the evaporation of large volumes of solvents is "chilling" the analytes. The entrance cone to the vacuum region of the instrument, with a diameter of less than 1 mm, is situated in an orthogonal position to the direction of the electrospray and bears a countercharge with respect to the charge of the capillary. Based on this principle, only ionized molecules enter the vacuum, whereas un-ionized and non-volatile molecules of the matrix remain outside. The high vacuum inside an LC-MS system is maintained by turbomolecular pumps (running with about 100000 revolutions per minute), which are substantially more powerful compared to pumps used in GC-MS. From the entrance cone, ions are directed into a quadrupole by "ion-optics", where a specific radiofrequency pattern allows just one single defined mass-to-charge ratio of ions to pass to the detector, as in GC-MS. Commonly, analyte molecules acquire one proton during atmospheric pressure ionization forming a "quasi-molecular" ion ($[M+H^+]$) but also cluster formation with sodium, potas-

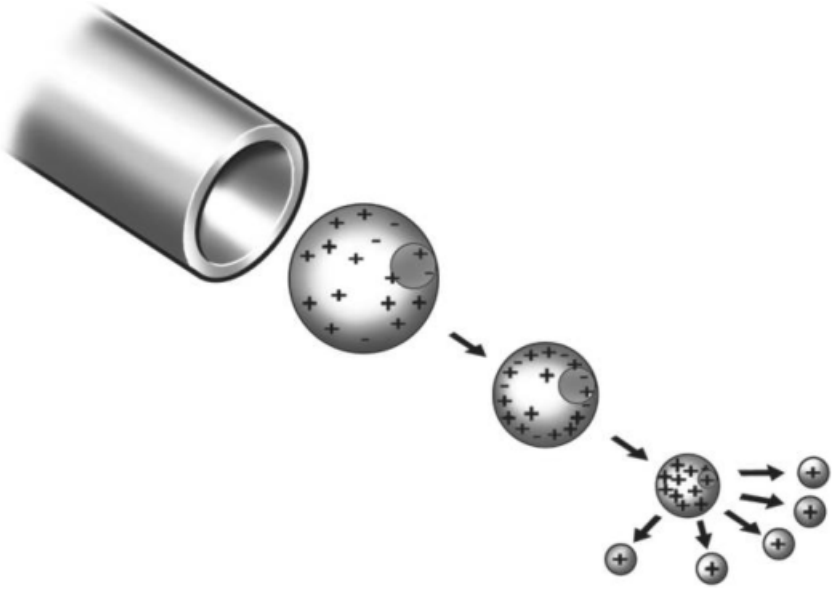

Figure 1 Scheme of the principle of electrospray atmospheric pressure ionization.

sium, or constituents of the mobile phase as ammonium or formiate ions can occur. Electrospray ionization represents a technique of "soft" ionization since usually the intact analyte molecule is generated in contrast to the disintegrating ionization in GC-MS.

An alternative technique to electrospray ionization is atmospheric pressure chemical ionization (APCI), where a corona discharge near the eluent spray transfers a charge to the droplets. APCI is in general better suited for the ionization of less polar compounds.

Single-stage MS

The first systems that combined HPLC with mass spectrometric detection involved one single quadrupole analyzer as described above ("single-stage MS"). These instruments offer the possibility to detect compounds lacking molecular properties that are the prerequisite for conventional HPLC detection techniques, such as UV absorption (*e.g.*, by an aromatic structure), fluorescence (as potentially found with conjugated double bonds), or characteristic electrochemical behavior. Furthermore, due to the detection according to mass-to-charge ratios, the specificity of these single-stage systems is superior to conventional techniques of HPLC detection. However, given the extreme complexity of biological samples with many completely different compounds of identical molecular mass and the general possibility of multiple charging of analytes in electrospray ionization (*e.g.*, a molecule with a molecular mass of 20000 that has accepted 10 protons has an m/z of 200 just like a singly-charged molecule of a molecular mass of 200), single-stage LC-MS still requires baseline chromatographic separation of relevant compounds if applied to samples of biological origin. For application in the clinical laboratory, therefore, the same practical problems in handling apply to single-stage LC-MS as is the case for conventional HPLC techniques. For application with GC the principle of the single-stage quadrupole technique is sufficiently specific for biomedical analyses due to the enormous separation capacity of capillary gas chromatography, which HPLC lacks.

Tandem mass spectrometry (TMS)

The essential step in the development of LC-MS as a routine technique for the clinical laboratory was the introduction of collision-induced dissociation (CID) with a second quadrupole for analysis of the fragments generated in this way ("tandem"-MS): In these instruments, a first quadrupole selects the molecular ("parent") ion of the respective analyte, which is then directed into a collision cell; here a very low flow of argon enters as the collision gas. By collision with these argon molecules the parent ions are disintegrated into several typical so-called "daughter" or "product ions". These daughter ions can then be scanned according to their respective m/z ratio by a second quadrupole positioned behind the collision cell within 1 second or less (Figure 2). Whereas in GC-MS a fragmentation "fingerprint" is generated on ionization by electron impact itself, in LC-TMS a "fingerprint" spectrum arises from controlled disintegration of selected intact parent ions within the collision cell.

By analogy to the selected ion recording mode of GC-MS for quantification with LC-TMS, usually the second mass spectrometer is set to the m/z ratio of one characteristic daughter ion of a respective parent ion (multiple reaction monitoring, MRM). Such "transitions" are usually given as, for example, "931>864", with 931 the mass-to-charge ratio of the parent ion, and 864 the mass-to-charge ratio of the production ion (mass-to-charge ratios are given for the compound sirolimus as an example).

Since the collision cell in principle represents a small quadrupole on its own instead of the term "tandem mass spectrometer", the term "triple stage mass spectrometer" can be used synonymously.

Using the principle of tandem configuration of two quadrupoles the specificity of LC-MS was increased substantially compared to single-stage systems, fully compensating for the limited separation capacity of HPLC compared to GC. Once these LC-TMS systems became available, a widespread use in the field of pharmacokinetic research studies by commercial laboratories began (1); within a few years LC-TMS replaced conven-

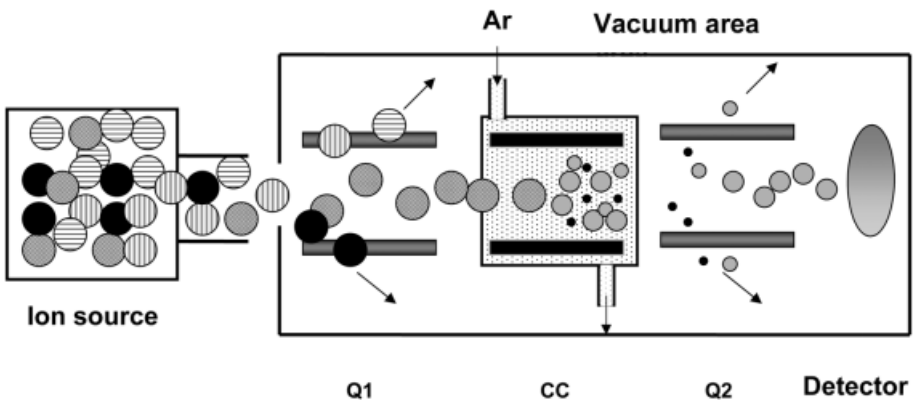

Figure 2 Scheme of the main elements of a tandem mass spectrometry system. In quadropol 1 (Q1) ions are filtered according to their mass-to-charge ratio (m/z) and a respective parent ion is selected; the selected ions undergo collision-induced decomposition in the collision cell (CC); in quadrupole 2 (Q2) the fragment ions are filtered according to their mass-to-charge ratio and a specific ion is selected, which is finally detected by the photomultiplier detection system.

tional HPLC and GC-MS techniques almost completely for these purposes by the end of the '90s, especially since the highest attainable analytical standard is generally required in studies addressing drug licensing.

Contemporary LC-TMS systems are bench top instruments with a footprint of approximately 1 × 1.5 meters. At present three manufacturers offer LC-TMS systems that are applicable in the clinical laboratory (Applied Biosystems, Foster City, CA, USA; Micromass, Manchester, UK; ThermoFinnigan, San Jose, CA, USA) with list prices ranging from about 200000 to 500000 Euro. The systems are comprised of user-friendly software packages that control the mass spectrometers, as well as the HPLC module and the autosampler and integrate all functions of data acquisition and quantification.

LC-TMS offers the possibility to skip between multiple MRM traces in time steps of fractions of seconds; up to eight parallel MRM traces can usually be analyzed without loss of sensitivity but potentially up to 20 traces can be acquired simultaneously. A typical LC-TMS chromatogram is given in Figure 3.

As shown in Table 1, LC-TMS offers essential advantages compared to both GC-MS and HPLC with conventional techniques of detection despite some specific limitations that are discussed below.

Method Development with LC-TMS

Mass spectrometric conditions

The development of an LC-TMS method is usually very straightforward compared to the development of conventional HPLC methods or even of immunoassays. The first step is the continuous infusion of a solution of the pure analyte substance into the mass spectrometer to optimize the conditions of ionization, which usually takes a few minutes. When the parent ion of the re-

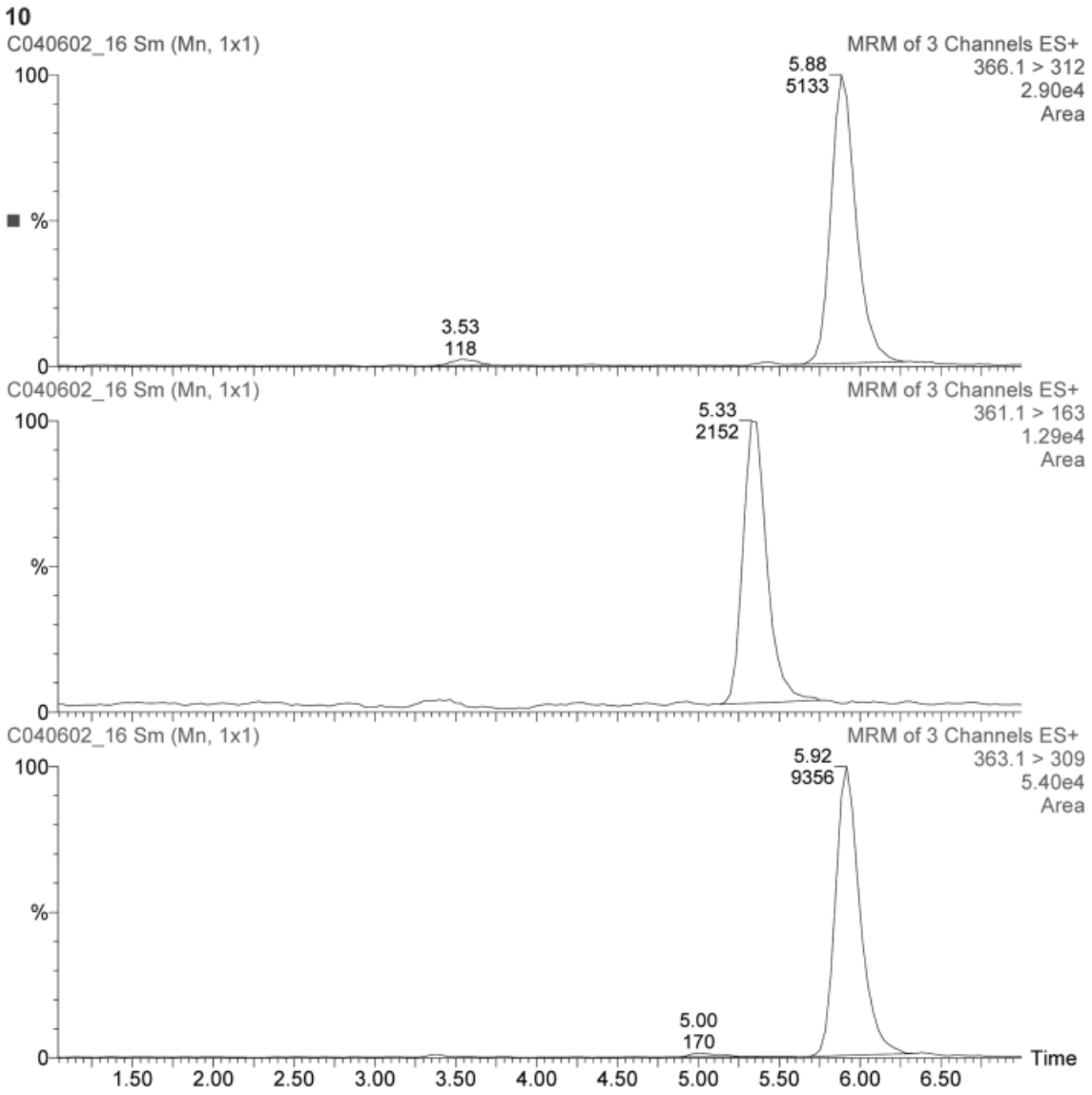

Figure 3 Typical chromatogram of a liquid chromatography tandem mass spectrometry method: determination of cortisol and cortisone with tri-deuterated cortisol as the internal standard (16). The respective parent and product ions are 366.1>312 for tri-deuterated cortisol, 361.1>163 for cortisone, and 363.1>309 for cortisol. Note the co-elution of cortisol and tri-deuterated cortisol.

Table 1 Characteristic features of liquid chromatography-tandem mass spectrometry, gas chromatography-mass spectrometry and conventional high-performance liquid chromatographic techniques.

	LC-TMS	GC-MS	Conventional HPLC
Prerequisite for detection of specific analytes	Efficient ionization of most analytes is possible irrespective of their molecular structure; no limitation by molecular mass.	Limited molecular mass (< 800); analytes have to be thermically stable and volatile per se or by derivatization.	For detection chromophores (*e.g.*, aromatic structures) are necessary for UV-detection, conjugated double bonds for fluorescence detection or characteristic redox properties for electrochemical detection.
Sample preparation	Mostly limited to rough removal of matrix proteins. No need for derivatization (most assays).	Complex and laborious; since the entire GC-eluent enters the high-vacuum region residual matrix compounds cause spoiling of these parts; cleaning requires venting of the system and is very laborious. Need for derivatization of the analytes, therefore typically sample preparation in several steps. Moreover, the completeness of derivatization in individual samples is always speculative.	Mostly complex and laborious; due to the limited resolution capacity of HPLC, analytes of interest have to be concentrated and separated specifically by sample preparation for example using solid-phase extraction cartridges.
Chromatographic separation	Very limited chromatographic separation needed since several multiple reaction monitoring traces can be detected in parallel by alternating MS-acquisition. Co-elution of several compounds does not hinder quantitative analysis.	High intrinsic resolution capacity of GC is necessary since usually the quantified fragments are merely characterized by one mass-to-charge ratio. In biological samples, however, this offers very limited specificity. GC-tandem MS systems exist but are not widely used.	Due to the poor specificity of all detection principles used for conventional HPLC (UV-VIS, electrochemical, and fluorescence detection), minute base-line separation of analytes is necessary; since the resolution capacity of LC is very much limited compared to GC; often complex mobile phase mixtures with specific gradient programs are necessary.

spective analyte is generated at optimum intensity a disintegration spectrum of that parent ion is recorded applying the collision gas. From this disintegration spectrum an intensive product ion is selected for multiple reaction monitoring; accordingly the collision energy and few parameters of the ion optics and quadrupoles are tuned to obtain maximum product ion intensity. Usually a standard mobile phase can be used for the mass spectrometric optimization of most analytes (for example a mixture of acetonitrile and water-containing ammonium acetate or formic acid as a proton donor). However, in some cases ion yield can be improved by modification in the composition of the mobile phase. After defining the analyte-specific pair of parent ion and product ion ("transition"), the sensitivity is tested by injection of the pure compound solution from a sample loop at decreasing concentrations into the instrument. With these few steps the mass spectrometric method set-up for a single compound is finished and is then performed in the same manner for all compounds that are intended to be quantified with one method. As the next step sample preparation and chromatographic conditions are elaborated.

Sample preparation

Sample preparation is necessary for LC-TMS analyses of biological samples since introduction of crude plasma or urine into the atmospheric pressure ionization source could inhibit the ionization of analytes of interest ("ion suppression") and would cause rapid spoiling of the ion source.

Depending on the concentration range of target analytes simple protein precipitation (for example with acetonitrile) may be sufficient to obtain acceptable results for many analytes. Lower target concentrations require larger sample volumes to be injected. However, residual matrix compounds present after mere protein precipitation will limit the lifetime of the analytical columns. Furthermore, co-elution of residual matrix compounds together with the analytes of interest may limit the sensitivity of detection by ion suppression

when mere protein precipitation and limited chromatography is applied. Ion suppression may be minimized by extended analytical run times that – on the other hand – limit the throughput of the expensive instrument (2). For these reasons in many applications liquid-liquid or solid-phase extraction is favorable. The latter approach can be automatized easily either by off-line sample handling systems with disposable cartridges or by on-line solid phase extraction with permanently used extraction columns and column-switching technique for on-line transfer to analytical chromatography (3–5). Both liquid-liquid and solid-phase extraction may include a concentration step and can thus increase the sensitivity of a method.

Chromatography

Despite the high (seemingly inherent) specificity of TMS, some degree of further chromatographic clean-up of the analyte after sample preparation is reasonable in order to separate the analyte of interest, especially from more hydrophilic conjugate metabolites. Such conjugates can potentially disintegrate during atmospheric pressure ionization ("in-source transformation") and can cause falsely high signal of the actually unconjugated compound if chromatography does not resolve between conjugated and unconjugated compounds (6, 7). Chromatography also can improve the ionization yield of the compounds of interest since "ion-suppression" may occur if the analytes co-elute with residual compounds of the sample matrix. Chromatographic run times of about 3–5 minutes are appropriate. Due to the high specificity of TMS and the possibility to acquire several MRM traces simultaneously, however, time-consuming and cumbersome optimization of the HPLC conditions (eluents, gradient programs, column materials, temperature, flow rates) with the goal of baseline resolution of all relevant compounds is not necessary as is the case with conventional nonspecific techniques of HPLC detection. Co-elution of relevant analytes is useful rather than being a problem. For some applications – depending on target analyte concentration – chromatography can indeed be omitted completely as for neonatal screening for inherited errors of metabolism (8).

Internal standards

LC-TMS principally requires the use of internal standards; solutions of these compounds are added to samples and calibrators of a series strictly in the same quantity, and quantification is based on the ratio of the peak area of the target compound to that of the internal standard trace (= response) in samples and calibrators. Ionization conditions and ion yield often vary within seconds with atmospheric pressure ionization; it is the essential requirement for an internal standard compound that this "undulation" of ionization efficiency has equal impact on the target analyte and the internal standard molecule, respectively; therefore, both compounds should be as similar as possible with regard to their molecular structure. This is given best in stable isotope-labeled internal standard compounds where three or more hydrogen atoms of the target analyte are replaced by deuterium or carbon-12 atoms are replaced by carbon-13 atoms. Corresponding to the number of stable isotope atoms introduced into an internal standard molecule, the molecular weight is higher by one atomic mass unit compared to the naturally occurring compound. However, the chemical behavior during extraction, as well as the properties of ionization, are almost identical to that of the unlabeled analyte. Consequently, methods employing stable isotope-labeled internal standards offer highest precision and accuracy. Alternatively to stable isotope-labeled internal standards, molecular homologues of the target analytes may be used (such as desmethyl homologues), but the applicability has to be validated thoroughly. Indeed, the availability of suitable internal standards is crucial for the development of an individual LC-TMS method.

Applications of LC-TMS

Neonatal screening

The first important step of LC-TMS technology into clinical medicine was the application to newborn screening of inherited metabolic disorders. In 1997, Rashed *et al.* (8) described a multi-method for the quantification of amino acids and acylcarnitines in dried blood spot samples of newborns. The method employs simple loop injection without chromatography and allows the analysis of up to 1000 samples with a single instrument per day (9). Using this approach the early diagnosis of a large number of inborn metabolic diseases has become possible that are missed by the current conventional screening merely for phenylketonuria. Namely, rare errors of amino acid metabolism (such as maple syrup disease), defects of fatty acid oxidation (such as carnitine deficiency), and errors in the metabolism of organic acids (as methylmalonaciduria) (10, 11) are missed. The results of LC-TMS screening offer the possibility to initiate specific diets and to prevent severe disease manifestation with mental retardation or sudden death in identified children. This extended screening panel based on LC-TMS is currently being applied in a growing number of regions worldwide and experiences continuously widen its use at present. Since severe lifelong disability can be avoided by using these screening programs, they are highly cost-effective despite the fact that thousands of analyses must be performed to detect one affected child. The diagnosis of phenylketonuria, as one of the most frequent inborn metabolic errors, is improved substantially by TMS compared to conventional chemical methods since in TMS the more reliable phenylalanine/tyrosine ratio is determined; consequently, the recall rate to initiate further diagnostic work-up has been reduced substantially by TMS.

Usually neonatal screening investigations are performed in few centers for large populations.

Sirolimus

For applications beyond newborn screening, LC-TMS systems were introduced into medical laboratories from the end of the '90s on, mainly, however, for research purposes. A further important step in the development of LC-TMS as a routine clinical chemistry technique was the therapeutic drug monitoring of the novel immunosuppressant sirolimus (syn. rapamycin). For the prevention of organ rejections after transplantation this drug offers important advantages over conventional immunosuppressants such as the calcineurin-inhibitors cyclosporine or tacrolimus. Most importantly, nephrotoxicity is absent; moreover, sirolimus exerts anti-proliferative properties and may thus prevent graft-vessel disease after heart transplantation and nephrosclerosis after renal transplantation. During clinical studies for approval of the drug the need for dosage individualization according to whole blood drug concentrations became evident. This is due to poor and variable absorption and to the inter-individually heterogeneous metabolization of sirolimus. During these studies an automated immunoassay for the quantification of sirolimus was available, however, this test was withdrawn by the manufacturer before approval of the drug. Therefore, alternative methods were the prerequisite for the clinical introduction of sirolimus. Since sirolimus has a very poor UV absorption conventional HPLC techniques are cumbersome and hardly applicable for routine drug monitoring. LC-TMS in contrast proved to be excellently suited for the quantification of sirolimus in whole blood (5, 12). With the availability of these analyses at the site of transplantation centers the introduction of sirolimus became possible in 2001. The very favorable clinical experiences with sirolimus now suggest that it will become the essential standard drug in transplantation medicine. Indeed, at present the need to monitor lifelong sirolimus treatment in transplant patients is the most important single reason to equip laboratories of tertiary care hospitals with LC-TMS systems.

General characteristics of LC-TMS applications

Once available in the setting of these respective tertiary care clinical laboratories, the specific options of LC-TMS for routine clinical chemistry applications now become evident. This is documented by a continuously growing number of published method protocols for clinically relevant analytes like cyclosporine, tacrolimus (4), urinary catecholamines (13), and metanephrines (14), busulfane (15), or porphobilinogen (16), which each offer substantial advantages over so far available methods.

In general, LC-TMS is highly attractive for target analytes where

i) sufficiently specific test antibodies cannot be generated for use in immunoassays (*e.g.*, biogenous amines such as catecholamines; vitamins (17); porphyrins and precursors; endogenous metabolites such as asymmetric dimethyl arginine (ADMA) (18); 17-α-OH-progesterone (19); dihydrotestosterone; conjugate metabolites of steroid hormones);
ii) several related compounds are to be quantified simultaneously in one analytical run (*e.g.*, typically co-administered drugs like sirolimus and tacrolimus (4) or an active drug compound together with an active metabolite such as itraconazole and hydroxy-itraconazole, or a hormone together with its pro-hormone such as cortisol and cortisone (20)).

Therapeutic drug monitoring

Conventional HPLC techniques are usually considered highly specific, but even given a symmetric peak shape, it must always be suspected that drug metabolites co-elute with the target drug compound. Many drugs undergo extensive metabolism, especially by the hepatic cytochrome P450 system leading to a variety of often poorly characterized metabolites. Only very rarely are these metabolites available as pure substances to allow the conclusive investigation of the analytical specificity of respective HPLC or immunoassay methods. With LC-TMS, however, the probability that a multiple reaction monitoring signal, characterized by the molecular mass of the parent ion and the mass of a product ion, is generated by an inactive metabolite of a respective drug, by a different drug, or by an endogenous compound, principally exists but is very low. Due to this specificity, LC-TMS represents to date the ideal technology for therapeutic drug monitoring.

In general, it is probable that therapeutic individualization according to drug concentrations in blood will gain further importance in the future. In the case of the relatively old drug ribavirin, for example, it became evident from recent research that target concentration-tailored dosage regimens essentially improve the therapeutic outcome in hepatitis C infection (in combination with interferon (21)). A respective LC-TMS method employing a stable isotope-labeled internal standard has been described (22). It is very likely that in many other drugs for which standard dosages are usually applied, individualization will improve therapeutic outcome, such as with antidepressants, anti-infectives in intensive care patients, anti-neoplastic drugs, or retroviral protease inhibitors.

Reference methods

In addition to the routine application in clinical laboratories, LC-TMS has the potential for the development of clinical chemistry reference methods for low-molecular weight analytes (3, 19). Classically, GC-MS has been used for this purpose. These conventional methods, however, are very laborious and their use is restricted to small investigational series. It is a more important limitation of GC-MS reference methods that they involve derivatization and the completeness of derivatization and the stability of derivatives in individual samples is always speculative. Therefore, it is likely that the LC-TMS will be preferred for the development of reference methods in the future.

Despite the specific limitations discussed below, a majority of clinically relevant low-molecular weight compounds can be subjected to LC-TMS analyses, whereas only a minimal proportion is analyzable with GC-MS.

Most importantly, this technique is limited to molecules below a mass of about 800 and to thermostable, apolar compounds. A further substantial difference between GC-MS and LC-TMS is the minimal extent of maintenance that is required by the latter technique. Methods are far more rugged compared to conventional chromatographic techniques and the practicability in a routine setting is substantially improved. For these reasons it is most likely that in the coming years LC-TMS will gain wider use in clinical laboratories than GC-MS ever did.

The technique of LC-TMS is highly versatile, and used complementary to immunoassays it will extend the analytical spectrum of the clinical laboratory for important analytes. LC-TMS will enable a higher degree of independence from commercial assay manufacturers for the clinical laboratory with respect to the selection of assays offered to clinicians and with respect to the quality and cost of the products. In Table 2, typical features of LC-TMS technology are compared with immunoassay applications.

The costs for the implementation of LC-TMS technology are indeed high but roughly in the same range as the costs of contemporary immunoanalyzers; a certain decline in the price of LC-TMS instruments – as seen with GC-MS systems – can be expected in the coming years but the systems will remain substantially more expensive than conventional HPLC systems.

Personal Experiences

In our laboratory, an LC-TMS system has been in use since 2000 mainly for therapeutic drug monitoring of sirolimus and other immunosuppressants. At a continuously high workload the instrument and the method (5) have proven to be extraordinarily rugged, with monthly coefficients of variations below 5%. The specific requirements of a clinical laboratory are fully met by the system; in particular no malfunction occurred that would have delayed reporting of the results that are essential for therapeutic decisions. The only regular maintenance procedure that is necessary at an interval of several months is cleaning of some parts of the ion source, which requires about 1 hour without venting the system and the exchange of HPLC columns after several thousands of analyses. The system is run by ordinarily trained medical technicians guided by a physician. Running costs per analysis are about 2.5 Euro compared to typical costs of 15 to 30 Euro for immunoassay tests for the quantification of immunosuppressants.

Limitations of LC-TMS

The sensitivity of LC-TMS systems still presents some limitation. In our experience with an instrument built in 1999, for most analytes a detection limit of about 1 µg/l can be achieved, which is sufficient for monitoring of most drugs and the analysis of important hormones. Sample preparation procedures that include a concentration step (*e.g.*, evaporation) may lower the limits of detection. However, compounds like plasma metanephrines are still a challenge for LC-TMS method development but technical improvements of the instruments will most probably increase the sensitivity of the systems.

Table 2 Characteristic differences between LC-TMS and immunoassays.

LC-TMS	Immunoassays
Very straightforward and versatile assay development (if pure substance and appropriate internal standard compounds are available)	Assay development with production of antibodies is hardly possible for clinical laboratories; therefore complete dependence on assay manufacturers with respect to assay development and analytical performance
Quantification of multiple analytes within one analytical run is possible (*e.g.*, main drug compound and metabolites or typically co-administered drugs); very high specificity	Determination of merely one analyte; often limited specificity and relevant cross-reactivity with related compounds (*e.g.*, inactive cumulant drug metabolites)
Limited sample throughput. Typical run time about 5 min; technical perspective of multiplexing with substantially increased throughput. At present most assays include manual steps in sample preparation but offer the perspective of full automation	In automated assays high sample throughput (up to or even above 100 tests per hour) without manual handling steps
High costs for instruments, very limited running costs (vials, solvents, standards, columns)	Costs for instruments (immunoanalyzer) often lower compared to TMS but typically very high running costs per test for reagents and services
Sensitivity of currently used instruments for most analytes limited to about 1 µg/l if no concentrations steps are included in the sample preparation	Often very high sensitivity (*e.g.*, 10 ng/l for estradiol)
Best suited for measurement of small molecules	Best suited for measurement of macromolecules (*e.g.*, proteohormones)

The specificity of LC-TMS is in general very high, however, it should principally be questioned; it can be limited by the already mentioned effect of in-source transformation. Nonspecificity may also arise, for example if active and inactive metabolites of a drug have identical molecular masses but groups of the molecule are present at different sites and these isomers also share common fragments in collision-induced disintegration. Multiple-charged macromolecules from the sample matrix may generate a variety of multiply-charged product ions and thus potentially the MRM transition of an analyte of interest may be matched. However, in these cases – if applying appropriate chromatography – atypical and distinguishable peaks are generated from multiply charged macromolecules.

It is not the case that virtually all low-molecular compounds are ionizable by atmospheric pressure ionization. Rather apolar compounds may be ionized more efficiently by use of APCI instead of the more widely used electrospray ionization (ESI). In all available systems, it is possible to switch between APCI and ESI within minutes. However, at present, with chemical ionization as well for analytes like cholesterol epoxides, no sufficient ionization is achievable to allow quantification at naturally occurring concentration levels. A further example for low ionization yield is methylmalonic acid, where a simple derivatization is necessary to achieve sufficient ionization (23). In some compounds, ionization is efficient but collision-induced disintegration fails to generate characteristic product ions for MRM, especially if sodium adducts of the respective analyte are dominating.

One potentially important advantage of GC-MS over LC-TMS is the fact that ionization and disintegration are achieved in GC-MS following worldwide standardized conditions, namely, electrons that are accelerated with 70 eV and helium as the carrier gas. Therefore, it was possible to generate GC-MS spectra libraries that can be searched electronically for unknown compounds in clinical samples (like unexpected poisons) and may thus allow their identification. This is of essential importance in legal medicine and toxicology. In LC-TMS, in contrast, the specific architecture of a source, a number of about 20 instrument settings and potentially the mobile phase, have an impact on ionization (possibly with formation of adducts) and disintegration. Therefore, it is questionable whether disintegration spectra libraries like for GC-MS can ever be established for LC-TMS to allow identification of unknown compounds.

The possibility to develop an LC-TMS method critically depends on the availability of appropriate internal standard compounds and this is not given in all analytes of interest at present. For example, specific monitoring of the active metabolites of azathioprine, the mono-, di-, and triphosphates of 6-thioguanine, would be clinically highly relevant but so far no internal standards are available for this purpose.

Complementary MS Techniques

It is the intention of this article to summarize concisely the basic characteristics of LC-MS applications that are most relevant for clinical laboratories. In fact, beyond quantification applying multiple reaction monitoring, LC-TMS offers further analytical possibilities for structural elucidation of compounds like the so-called survey scan, the precursor-ion scan, and the neutral loss scan that are not described here.

A complementary mass spectrometric technique that is currently a most important and versatile research tool in "proteomics" but also has potential for routine application is MALDI-TOF. This represents the combination of a laser-based ionization technique (matrix-assisted laser desorption ionization, MALDI) from solid samples like dried serum spots and the very powerful principle of time-of-flight (TOF) mass spectrometry of macromolecules. By using specialized transformation software, sequencing of proteins and amplified DNA is possible with this technique (in principle, however, this is also feasible with electrospray LC-TMS).

In so-called ion-trap instruments – sharing the principles of atmospheric pressure ionization with LC-TMS – selected parent and product ions are kept as a cloud within a radiofrequency field. Disintegration of selected ions is performed discontinuously by radio frequency impulses and thus potentially "MS^n in time" with several generations of product ions is possible instead of MS/MS ("MS^2 ") "in space" as in LC-TMS, enabling even more specific analyses despite lower instrument costs compared to TMS systems. However, the discontinuous manner of signal generation in ion-trap instruments limits the number of data points generated "over" an LC peak, potentially resulting in limited precision of the assays.

Future Developments

At present, calibration materials, quality control materials, internal standard compounds, equipment and supplies for sample preparation, and chromatography are not yet commercially available as complete kits. It is likely, however, that the diagnostic industry has recognized LC-TMS as an up-coming routine clinical chemistry technique with potentially widespread use and will provide the market with appropriate products soon, most importantly with a variety of stable isotope-labeled internal standard compounds.

Further technical development of LC-TMS instruments will probably result in increased sensitivity, especially enabling the quantitative analysis of endogenous compounds circulating in very low concentration ranges. Furthermore, it seems likely that LC-TMS will be used as a technological platform for the development of automatized complete analyzer solutions combining sample preparation and mass spectrometric analysis as selective random-access analyzers with specific application fields complementary to clinical chemistry and immunoanalyzers.

Conclusion

In summary, LC-TMS is an important new technology substantially extending the analytical spectrum of the clinical laboratory. LC-TMS is not merely a powerful new detection principle for HPLC (as applies for single-stage LC-MS), but represents an analytical principle on its own with HPLC used for sample preparation. LC-TMS offers highest analytical versatility and productivity combined with excellent practicability in a routine setting and will probably gain far more widespread use than GC-MS in the future.

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