LC-MS STRATEGIES IN MONITORING THE RESPONSE TO DIFFERENT THERAPY

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Abstract
Therapeutic drug monitoring is an essential tool for a positive management therapy. The preferred laboratory strategy is immunoassay. Due to the cross-reactivity to other components, alternative approaches, such as LC-MS, are continuously under investigation. The paper described briefly the main aspects regarding LC-MS apparatus and technique and recent applications on therapeutic drug monitoring, other than already extensively described applications as in immunosuppressing therapy monitoring.

Rezumat
Monitorizarea farmacoterapiei este un instrument esențial al unui management terapeutic pozitiv. Strategia de laborator preferată este imunoanaliza. Datorită reactivității încrucișate cu alți componente, alte metode sunt în continuă investigare, cum ar fi metoda LC-MS. Lucrarea descrie într-o manieră sumară principalele aspecte privind aparatul și tehnica LC-MS și aplicații recente ale acesteia în monitorizarea farmacoterapiei, altele decât cele descrise deja în literatura de specialitate, cum ar fi monitorizarea terapiei imunosuprensive.

Key words: therapeutic drug monitoring, LC-MS

Introduction
Many clinical strategies base their treatment decisions on subjective judgment, rather than quantitative measures. Clear evidences from several studies show that treatment decisions that are driven by quantitative monitoring are significantly better than subjective monitoring for improving patient outcomes [1].

International regulations and guides recommend monitoring of plasmatic concentrations in patients using drugs with narrow therapeutic index from antiepileptic, antiinfective, antiasthmatic classes or in patients under immune-suppressing therapy (cyclosporine, tacrolimus, sirolimus). Overdosing or subdosing risks can be avoided by pharmacotherapy monitoring. In case of chronic renal disease inadequate administration can
cause dose dependent toxicity, because it affects drugs absorption, bioavailability and distribution and it modifies renal clearance.

The optimal disease management is founded on a multidisciplinary approach in which analytical analysis of drug plasma concentration plays an important role. While some measurement tools may be more appropriate for the use in clinical trials, several simple and practical tools have been developed that are even suitable in a busy clinic. The liquid chromatography coupled with mass spectrometry (LC-MS) is still not used as a common technique in routine analysis in the clinical laboratory and the reasons vary from the high prices of the equipment to the know-how of operation. However, there are some medical areas in which LC-MS demonstrated already the utility, such as therapeutic drug monitoring (TDM) or toxicology and new application could already be added such as biomarker discovery [2].

The aim of this paper is to underline a few new aspects regarding applicability of the LC-MS on TDM, other than already extensively described applications as in immunosuppressing therapy monitoring, preceeded by a short description of the main aspects regarding LC-MS apparatus and technique featured for bioanalysis.

A few technical remarks on LC-MS technique

Liquid chromatography is a powerful and universally accepted technique that offers chromatographic separation of individual analytes within liquid mixtures. In LC-MS the analytes are subjected to an ionization and then are introduced into a mass spectrometer [2-4]. In the bioanalytical research LC-MS is the preferred analytical technique and used both for qualitative and quantitative biological determination or drug identification [5]. Its analytical power is compared sometimes with X-ray diffraction, in terms of quality of the analysis, and overcomes all other LC methods on sensitivity, selectivity and high-throughput analysis, depending on the management of its multiple technical valences [6].

The link between the chromatograph and the detector in LC-MS is represented by the interface in which ionization occurs, usually at atmospheric pressure (Atmospheric Pressure Ionization – API). The most common interfaces are electrospray ionization (ESI) interface and chemical ionization (CI) interface, both operating at atmospheric pressure (AP). Other types of ionization: desorption ionization (DI), electron ionization (EI), atmospheric pressure photoionization (APPI), sonic-spray interface, laser spray interface etc.

In ESI the sample solution spray creates small droplets that carry charges induced by a potential difference between a nebuliser and an end-plate from a needle. The charge is maintained while the droplets become
smaller as the neutral solvent molecules evaporate, then protonation occurs to form highly charged sample molecules.

In APCI (Atmospheric Pressure Chemical Ionization), an aerosol of the sample solution is sprayed at atmospheric pressure into a heater in which a sharp metal pin held at high potential sustains a corona discharge. The action of the discharge on the solvent creates reagent ions that react with the neutral sample molecules to produce protonated ions of the molecule.

The mass analyzer, the main component of the mass spectrometer, contains an electric or magnetic field, or a combination of the two, which can manipulate the trajectory of the ion in a vacuum chamber. The most popular mass analyzer is the quadrupole (Q). A Q mass filter typically consists of four cylindrical electrodes with precise direct current and radiofrequencies voltages applied. The tandem Q mass filters (called as triple quadrupole QQQ) are referred to as Q1 and Q3, the additional Q2 quadrupole has no filtering effect and guides ions through the vacuum chamber.

A mass spectrometer with a single quadrupole is capable of working in two ways:
- full scan acquisition - the instrument detects signals over a defined mass range during a short period of time and generates the mass spectrum ions abundance versus m/z; it is used for qualitative analysis when the analyte mass is not known;
- selected ion monitoring (SIM) detection - the quadrupole filtering and monitoring only a specific m/z ratio; it is used for quantitative analysis.

The tandem LC-MS/MS allows unequivocally the identification of a substance in the sample. It is capable of working in another four ways:
- multiple reaction monitoring (MRM) - the first analyzer selects a parent ion from the first quadrupole, the selected ion suffers collision in another quadrupole via bombardment with gas molecules such as nitrogen or argon; at that moment, fragmentation occurs and the other mass analyzer in the third quadrupole selects a particular product “daughter” ion; the correlation between the parent and product ions offers information regarding the chemical structure;
- product ion scanning – the mass-selected precursor ion is induced to dissociate into product ions, which are then mass analyzed by a second analyzer from the mass of the precursor downwards; the result is a mass spectrum that contains signals for all the product ions formed from that selected precursor ion;
- precursor ion scanning – the second mass analyzer is set at the mass of the selected product ion, and then the first mass analyzer is scanned from that mass upwards;
neutral loss/gain scanning – it is a scan that determines, in a single instrument, all the parent ion mass/charge ratios which react to the loss or gain of a selected neutral mass.

Other types of mass analyzers are: magnetic sector, time-of-flight (TOF), quadrupole orthogonal acceleration TOF (Q-TOF) and ion trap (IT).

The selectivity of LC-MS/MS reduces the need for complete chromatographic resolution of individual components, allowing a shorter analytical run time and higher throughput [5]. Typically the tandem LC-MS/MS increases in selectivity and sensitivity and provides a more than 10-1000 fold improvement in the lower limit of quantitation (LLOQ) compared with traditional methods using ultraviolet or fluorescence detection.

**Sample pretreatment in bioanalysis**

It is well accepted that sample preparation was targeted as a rate limiting step in the overall procedure for bioanalysis.

There are two types of sample preparation, „off-line” when the procedure is performed before the introduction in the sampling disposer of the equipment and „on-line” refers to an approach of an instrument by first preparing the sample and then analyzing it in sequence. Automation is an increasingly essential tool in a bioanalytical research. Automated processes improve laboratory productivity and bring a degree of reproducibility and quality to the results that cannot be realized among different workers each performing the method manually [5-7].

Among the sample preparation methods for bioanalysis, a few are more common such as protein precipitation (PP), liquid-liquid extraction (LLE) and solid-phase extraction (SPE),

**Protein precipitation** eliminates in a very simple way the most important interferents from biological samples. The advantage refers to the low cost and simplicity of the procedure: adding the precipitant agent, mixing, centrifugation, supernatant sampling. Typical additives are: trichloroacetic acid (TCA), acetonitrile and methanol. PP could be automated into a 96-well plate format by means of a robotic liquid handler, obtaining at least a fourfold improvement in sample throughput on the LC-MS instrument, compared to manual protein precipitation procedures [2,8]. Dezadvantages: TCA could induce ion suppression, poor analyte recovery or unsufficient limit of quantitation, in some cases.

**Liquid-liquid extraction** is performed in the following steps: adding of the immiscible solvent to the aqueous sample (with or without pH modification), shaking, centrifugation, removing of the organic solvent, organic solvent evaporation, dissolution of the residue with a proper solvent [2]. Typical solvents are: dichloromethane, ethyl acetate, methyl t-butyl
ether, hexane, chloroform etc. The advantage refers to the selective partitioning and enrichment of the analyte. In comparison with PP, LLE can be time consuming and labour-intensive. There are applications in which LLE is performed in an automatic 96-well plate plate format [2,9].

Solid-phase extraction is performed in four stages: conditioning of the SPE material, applying the aqueous biological sample through the SPE cartridge or disk, washing the hydrophilic interferences with water or 5% aqueous acetonitrile, eluting the analytes [2,10]. Typical materials for SPE: silica, chemical modified silica (C8, C18 etc.), ethylbenzene-divinylbenzene or styrene-divinylbenzene copolymers, graphitized non-porous carbon, graphitized carbon black. It could be used in off-line mode, in 96-well plate format, on-line mode or turbulent-flow chromatography. In comparison with previous methods, SPE is more expensive, but enables significant analyte enrichment.

Therapeutic drug monitoring by LC-MS

A large number of monitoring assays are available within the clinical laboratory, and it is often difficult to predict when a particular method is suitable to be applied, that is why these methods must above all have to be simple and robust, and should not require extensive methodology. Immunoassays are often applied. However, due to the cross-reactivity to other components, alternative approaches, such as LC-MS, are continuously under investigation. As an example in this direction, in a recent article a LC–MS–MS method is presented for the qualitative screening of 238 drugs in blood samples [11]. After a two-step liquid–liquid extraction and C18 chromatography, the compounds were introduced into a triple quadruple mass spectrometer equipped with a turbo ion spray ion source operating in the positive ionization mode.

Some examples of drugs for which monitoring is very important are: digoxin, phenytoin, aminoglycoside antibiotics, theophylline, cyclosporine, HIV protease inhibitors, some cardiac agents, antiepileptic and antidepressant drugs. However, many other drugs are candidates for this therapy approach.

Immunosuppressive medicines are characterized by a specific pharmacokinetic profile which requires TDM (therapeutic drug monitoring). Immunosuppressive protocols are dominated by calcineurine inhibitors (cyclosporine, tacrolimus), which are used in combination with other immunosuppressive medicines (mycophenolate mofetil, sirolimus, corticosteroids, basiliximab). Many articles and book chapters were written on this subject, including analytical considerations [2,6,12,13]. The interpretation of the results from TDM is of great importance and must be
multidisciplinary considered that there are numerous factors of variability of patients and immunosuppressive medicines.

The therapy with antiepileptic drugs has to be also monitored. Phenytoin is widely used in the treatment of epilepsy and is effective against all types of seizures. No drug has greater need for therapeutic drug concentration monitoring and individualized dosing than phenytoin, even auto-induction should be considered during the treatment with phenytoin. In the recent years, there were written a few articles about LC-MS determination of phenytoin in human plasma.

Achim and col. described recently a LC-MS method for phenytoin concentration monitoring in plasma [14]. The procedure involves a simple PP (protein precipitation) from plasma with methanol. After centrifugation, the supernatant was injected into a C18 column, and eluted with a mobile phase consisting in a mixture of 2 mM ammonium acetate and methanol. Detection was performed in MRM (multiple reaction monitoring) mode using an ESI+ (electrospray ionization) mode. The monitored ion transition was 253.1→(182.1+225.1). The LLOQ was established at 2.0 μg/mL.

In another work, the procedure involves isolation of the unbound drug phenytoin from the drug/protein complex by ultrafiltration [15]. Liquid–liquid extraction was employed to extract the resultant ultrafiltrate. The analyte was separated on a reversed-phase column using isocratic mobile phase conditions and MS detection that yielded a run time of 1.5 min, enabling high throughput sample analysis. The LLOQ was 5.0 μg/mL.

A tandem mass spectrometry assay for the simultaneous determination of acetaminophen, caffeine, phenytoin, ranitidine, and theophylline in small volume pediatric plasma specimens was developed [16]. Sample preparation was performed by PP with methanol after addition of internal standard in plasma specimen. After chromatographic separation on a C18 column using gradient elution, analytes were detected using a QQQ mass spectrometer that was operated in ESI+ mode.

Other recent published method measures simultaneously zonisamide, lamotrigine, topiramate, phenobarbital, phenytoin, carbamazepine, carbamazepine-10,11-diol, 10-hydroxycarbamazepine, and carbamazepine-10,11-epoxide [17]. Sample pretreatment consisted of a single SPE (solid phase extraction) for all antiepileptic drugs in a 100-μL plasma sample. HPLC separation was achieved on a C18 column with a gradient mobile phase of acetate buffer, methanol, acetonitrile and tetrahydrofuran. Four internal standards were used. Detection was achieved by APCI-MS (Atmospheric Pressure Chemical Ionization - Mass Spectrometry) in SIM (selected ion monitoring) mode with constant polarity switching.
Antibiotics therapy is not a typical example for TDM. However, as it is well known, chronic renal insufficiency modifies renal elimination of drugs and raises problems for the achieving efficient dose and fluoroquinolones are a good clinical antibiotic option due to their large spectrum and bactericide activity, having a safety profile better than other antibiotics.

The main application area of LC–MS in the analysis of antibiotic and antibacterial compounds is the confirmation of identity in animal food products for human consumption at maximum residue levels, set by the regulatory authorities. However, in the past years there are many applications of LC-MS on TDM of fluoroquinolones and a few are presented below.

Ciprofloxacin is a synthetic fluoroquinolone used for the treatment of bacterial infections in many parts of the body. Bugge and col. [18] validated a LC-MS-MS method with a quantitative range of 25 to 10,000 ng/mL ciprofloxacin in human plasma, lomefloxacin, being the internal standard. Acetonitrile was used to precipitate plasma proteins. A part of the extract was evaporated and reconstituted in mobile phase. The LC-MS-MS analysis was performed on a C-18 column with an aqueous acetonitrile/acetic acid mobile phase. A mass spectrometer with heated nebulizer was used to monitor the eluent in positive ion MRM mode, ciprofloxacin (m/z 332→231) and lomefloxacin (m/z 352→249) both eluted at approximately 1.2 minutes. By applying this method, 600 samples per day could be analyzed.

A selective and sensitive high performance liquid chromatography–electrospray ionization –mass spectrometry method has been developed for the determination of balofloxacin in human plasma [19]. The sample preparation was LLE (liquid-liquid extraction), and chromatographic separation was achieved with a C18 column using a mobile phase of methanol–ammonium acetate. The limit of detection was 0.02 μg/mL.

Moxifloxacin was also determined from plasma by LC-MS-MS after SPE, lomefloxacin being the internal standard [20]. The method was based on the precursor and major product ion of the analyte monitoring on a QQQ mass spectrometer with ESI+ in the MRM mode. The limit of detection for the method was 50 pg/mL.

Ballesteros and col. [21] developed a method of determination of three widely used fluoroquinolones (norfloxacin, ciprofloxacin and ofloxacin) in human urine by LC-MS(ESI) method in SIM mode. The three fluoroquinolones studied and enrofloxacin, used as internal standard, were extracted from human urine samples by SPE. The method detection limit was about 10 ng/mL for the three fluoroquinolones.
**Other LC-MS applications in TDM**

Progesterone is suitable to be monitored during the treatment of infertility. A new simple, sensitive and selective LC/MS method for quantification of progesterone in human plasma was validated [22]. The analyte was eluted in 2 minutes on a C18 column under isocratic conditions using as mobile phase a mixture of formic acid 0.1% (v/v) and methanol. The detection of the analyte was in MS/MS mode using APCI+, m/z 315.2 → m/z 279.2. The sample preparation consisted in PP with methanol. The limit of quantification was 0.8 ng/mL.

Methadone pharmacokinetics has a large inter-individual variability. Recently, the methadone isolation efficiency by LLE and SPE was determined [23]. Different organic solvents (ethyl ether, ethyl acetate and hexane) or solvent mixtures and different SPE cartridges were tested. Methadone was quantified by an original LC-MS-MS method.

Due to the high potential of ivabradine to give adverse reactions on overdosing, but also considering the lack of therapeutic effect on underdosing, in a recent study, a fast HPLC-MS/MS method was developed, able to quantify ivabradine in human plasma after oral administration of 10 mg ivabradine, by applying a simple PP [24].

**Conclusions**

Selection of an analytic method for TDM usually involves an evaluation on chromatography and immunoassay techniques. There is no single correct or preferred assay technique and the best decision, based on technical, clinical, and economic considerations, will vary in different clinical and laboratory environments. The criteria to be used in sample preparation should aim to optimize factors such as compatibility with the instrument, recovery and high-throughput analysis. LC-MS-MS analysis after a simple protein precipitation and ESI mode could be taken into account as a high-throughput analysis in TDM in many cases.

**References**


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