LC/MS Methods for Small Molecule Biomarkers

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Abstract Small molecule biomarkers provide insight into metabolic pathways related to therapeutic treatment and can help explain the mechanism of action during drug development. LC-MS-MS method approaches parallel those for drug pharmacokinetic analyses; the optimization of detection, chromatographic system, and extraction are similarly important to achieving good quantitation. Endogenous presence of small molecule biomarkers presents a unique challenge for developing and validating an LC-MS-MS assay and several strategies are available to address it. Additional challenges for small molecule biomarker assays are sensitivity, ion suppression/matrix effects, and assay selectivity. Innovative solutions for overcoming these hurdles are discussed in this chapter.

Keywords Mass spectrometry · LC-MS-MS · Chromatography · HPLC · Extraction · Endogenous · Surrogate · Matrix · Sensitivity · Selectivity

Introduction

Biomarkers come in all shapes and sizes. A lot of emphasis has been placed on proteins and genetic processes, but small molecules can provide information not found with larger molecules with regard to metabolic pathways, pathology, causes, and potential treatment. In this chapter, the role of small molecule biomarkers will be discussed briefly, followed by what comprises a liquid chromatography mass spectrometric (LC-MS-MS) assay. The challenges inherent to LC-MS-MS biomarker analyses and some examples of current small molecule biomarker assays will also be covered.

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Drug Development—Efficacy

Metabolomics provides a vast number of potential biomarkers for measuring how well a pharmaceutical compound is working. Energy substrates and metabolites are monitored for changes in diabetics during treatment. Cholesterol and lipid levels provide feedback for the progress cardiac patients are making on lipid lowering medications. For new drugs under testing, these same markers are valuable tools for establishing how they are producing their effect as well as allowing a comparison with established treatment lines.

Drug Development—Safety

Biomarkers related to drug safety is a relatively new area in drug development with only a few recognized and validated protein compounds targeting renal and liver function. Small molecule safety biomarkers are of interest, but not well investigated. Any compound that may be linked to morbidity and mortality are candidates to be used. Blood lipids are an example of potential safety indicators for long-term effects related to cardiovascular disease.

Advantages of LC-MS-MS Measurements

Sensitivity

Tandem mass spectrometry brings extreme sensitivity to detection of analytes when coupled to HPLC applications. Background noise is greatly reduced by the selectivity of single ion mass monitoring (SRM or MRM mode), elevating the signal-to-noise ratio over less selective detectors.

Selectivity/Specificity

While signal-to-noise is increased, interferences are reduced through the filtering of all but the target mass ranges of interest. Comparisons of HPLC-UV and LC-MS-MS demonstrate the much shorter chromatographic run times of LC-MS-MS techniques [1].

Development of LC-MS-MS Assays

Mass Spec Optimization

Just as in the bioanalysis of an exogenous compound, endogenous biomarker analysis by LC-MS-MS first begins with the optimization of the mass spectrometer in order to detect the compound of interest. Mass selection (of precursor and product ions) and settings optimizations are the tasks involved in order to give the best response for a given amount of material. This can be accomplished through flow injection or constant infusion of the compound while monitoring in a scanning mode. Lens settings are ramped in order to find the optimal settings that promote the greatest signal for the masses found.

Liquid Chromatography (LC) Optimization

Of course the optimizing of the LC system provides an important aspect of the selectivity of the overall method by separating potential interferences from the analyte of interest through the orderly retention and elution from the LC column.

However, the selection of the HPLC parameters used in tandem with mass spectrometric detection can have a significant effect on sensitivity as well. Specifically, mobile phase composition and flow rate can either improve or inhibit the response of the analyte, depending on what additives and parameters are used. Generally, mobile phase additives that promote an easier evaporation (desolvation) when the liquid stream is nebulized in the mass spectrometer source, improves the transmission of ions to the mass spectrometer analyzer.

The decisions regarding the LC system for a given method will have an extensive effect on the sensitivity. Compounds that are very polar that retain poorly on a reverse phase column (C18) typically run at a mobile phase composition of 10 % organic solvent (methanol, acetonitrile) and 90 % aqueous solvent (water). The sensitivity of these compounds suffers under these conditions as the nebulized droplets from the LC flow struggle to evaporate and reduce in size. However, these same compounds are more retained on the polar ligands of normal phase or HILIC columns, and the mobile phase compositions at elution tend to be in the 80–90 % organic solvent range. The higher organic solvent composition makes it easier for desolvation in the mass spec source and improves sensitivity.

Some compounds show signal sensitivity to acid and base additives in the mobile phase as well. This may be due to ion pairing with the analyte, competitive protonation (in positive ion mode), or surface tension effects of the nebulized droplets in the mass spectrometer source. Regardless of the etiology, it is worthwhile to screen for these responses to mobile phase composition if sensitivity of the biomarker method is going to be one of the major challenges of development.

Sample Preparation Techniques

The simplest preparation technique is the direct injection of sample, presumably plasma or urine, into the LC solvent stream where it is cleaned up on-line through a trapping column or in-line solid phase extraction (SPE) [2]. This type of technique produces challenges in preserving the stability of the thawed, unaltered sample as it waits for processing in the sequence queue. However, it is the simplest form of manual preparation, as the sample is placed directly into the system where aliquot aspiration and processing are completely automated.

Most forms of sample preparation fall under the categories of sample dilution or protein precipitation (plasma samples), liquid–liquid (two-phase) extraction, or solid phase extraction (SPE).

Sample dilution of non-protein containing samples is a process where a quantitative aliquot of sample (urine) is combined with an internal standard and further diluted with a reagent compatible with injection. The advantages are that there is very little manipulation leading to little going wrong in the extract preparation. This technique is used for assays that have relatively high quantitative ranges where the detector signal can afford to be diluted.

Key Term 1

Internal Standard: A related compound added at predefined stage of the sample preparation process to provide a method for normalizing variations in analyte recovery, autosampler injection, and instrument response. The same amount is added to all samples within a batch and the ratio of analyte response divided by the internal standard response is used for regression analysis and quantitation of samples.

For protein containing samples (plasma, tissue homogenate), there is the potential for protein to precipitate as the diluted sample is introduced to the solvent stream of the LC system. This causes the HPLC column to clog and give high back pressure to the pumping system and can cause secondary chromatographic artifacts in the form of peak shape aberrations. Therefore, protein precipitation of these samples off-line, prior to injection, is performed as part of the extract preparation. The tertiary structure of the protein is forced to rearrange and fold in on itself by using a reagent, leading to insolubility and precipitation. The supernatant of the precipitated sample can be injected directly onto the LC system, or it can be evaporated to dryness and reconstituted in a more appropriate solvent. The reagents preferred for LC-MS applications are aqueous soluble solvents such as acetonitrile, methanol, and acetone. Strong acids such as trichloroacetic acid, perchloric acid, and trifluoroacetic acid are usually avoided as precipitating reagents due to their effects on ionization or their poor volatility in the instrument source.

Liquid–liquid extraction utilizes an immiscible organic solvent to form two layers of liquid separated by an interface. The analyte of interest starts out in the aqueous layer of the sample aliquot and diffuses across the interface until equilibrium is reached. The organic solvent is then removed and evaporated to dryness

before reconstituting for injection. Numerous buffers can be added to the sample to encourage a greater partitioning to the organic solvent. Different forms of mixing are used to accelerate the equilibration process. This type of extraction produces a very clean final extract, as a lot of interferences, proteins, and phospholipids are left behind with the aqueous layer. It also allows for a fast and easy process for concentrating the sample when additional sensitivity is required.

Solid phase extraction (SPE) is a technique where a mixed liquid sample is applied to a solid resinous sorbent contained in either a plastic cartridge or the well of a 96-well extraction plate. The sorbent may contain one of several types of chemical ligands that function to interact and retain compounds with certain functional groups or chemical properties. An SPE reverse phase sorbent will have affinity for lipophilic components of the sample. An SPE strong cation exchange sorbent will attract and retain compounds with basic functional groups. Once the analyte is adsorbed to the solid phase of the cartridge/well, other components of the sample can be rinsed away with selected wash reagents. When it is time to recover the analyte from the sorbent, an eluting solution is passed through the cartridge/well and the adhered compound releases and collects in a test tube or 96-well block. This eluate is often evaporated to dryness and reconstituted before injection, but on certain occasions it can be injected without evaporation. This technique can produce a very clean extract and is very effective for more polar compounds, which can otherwise be difficult to extract without significant loss of recovery.

Challenges of Biomarker Analysis by LC-MS-MS

Most of the challenges with small molecule biomarker analysis relate to their being endogenous to the control matrix that would otherwise be used for calibration and quality control samples of targeted concentration. The underlying native concentration of the compound makes it difficult to determine the accuracy of any preparations used for quantitative assessments.

Calibration samples that are fortified to a target concentration will be biased by the endogenous level present in the lot of control matrix used. This can be subtracted from each sample to give an adjusted regression of the calibration curve, but it must be recalculated with each change in control matrix lot, and can often raise a question about the true quantitation/detection limit of the analysis if the background contribution is high compared to the lowest target concentration. The run–to-run variability related to background subtraction is the reason other alternatives are just as popular for addressing the quantitation of endogenous compounds.

To avoid background subtraction of endogenous compounds, calibration samples need to be prepared in a comparable matrix (to the experimental samples) which is free of the analyte being measured. This can be achieved in a few different ways. Matrix lots can be screened for suitably low levels (below detection or quantitation) of the endogenous compound. The control matrix can be altered to remove or destroy the endogenous compound before using for calibration sample

preparation. An alternative species can be used as a control matrix source if the presence of the analyte is species dependent. Also, a proxy matrix can be prepared that is free of the endogenous compound [3, 4]. In all of these cases where a substitute matrix is used, testing to ensure comparable accuracy is achieved between the authentic matrix and the surrogate by way of standard addition to the authentic matrix and measuring the accuracy of the addition [3, 5]. Parallelism of calibration curves prepared in the authentic and surrogate matrix provides similar support for the alternate matrix [6].

Determining the quantitative range of a small molecule biomarker assay can sometimes be difficult compared to selecting the upper and lower limits of a pharmacokinetic assay. When an assay is used for drug development purposes, more is needed than simply having a positive or negative response from the biomarker. A quantitative measure is typically needed in order to be able to apply statistical analyses when looking at effectiveness of a treatment. This requires measuring baseline levels under normal conditions as well as under conditions of upregulated or downregulated influence.

Where an inhibited response is expected under treatment, sensitivity for the biomarker then becomes an issue. Measures to optimize sensitivity, such as low flow rates, high organic composition of mobile phase, increased sampling size, or in extreme cases derivatization of the analyte may need to be employed. Where an enhanced response is expected under treatment, determining the highest responses becomes the challenge in order to set the upper limit of the assay. With larger dynamic calibration ranges comes issues with linearity of the detector and contamination/carryover from automation equipment and injection instrumentation.

Key Term 2
Derivatization: A process used in analytical methods to change the molecular structure of a compound with a reagent that reacts with one or more functional groups on the analyte. Examples of derivatized products are esters, amides, oximes, and hydrazones. This process can help change the characteristics of the compound for the purpose of analysis. For example, a derivative may be employed in order to improve the chromatographic behavior of a compound.

Current Small Molecule LC/MS Methods

Existing methods for small molecule biomarker analysis by LC-MS are numerous, and the details of these methods are not always available through publication. Table 1 includes a listing of methods found across several therapeutic areas. Additionally, selected methods are discussed with the details of how the inherent difficulties of the compounds were overcome.

Table 1 Other small molecule biomarkers by LC-MS

Disease area	Disease	Compound	Matrix	Reference
Neurology	PD, PAF, Depression	DOPAC, DHPG	CSF	[23]
		Serotonin, 5-HIAA	CSF	[24–26]
Oncology	Melanoma	5-S-Cysteinlydopa	Plasma	[27]
	Intestinal carcinoid tumor	5-HIAA	Urine	[2]
Metabolism	Fatty acid amide hydrolase inhibition	Ethanolamides	Plasma	[28]
	In vivo CYP3A4/5 activity	4β-Hydroxycholesterol/ cholesterol	Plasma	[3]
Cardiology	Atherosclerosis	Eicosanoids	Plasma	[4]
Environmental	Toluene exposure	Hippuric acid	Urine	[29]
Pulmonary Disease	COPD	Desmosines	Plasma, urine	[30]

Abbreviations DOPAC = dihydroxyphenylacetic acid, DHPG = dihydroxyphenylglycol, 5-HIAA = 5-hydroxyindoleacetic acid, PD = Parkinson’s Disease, PAF = pure autonomic failure, COPD = chronic obstructive pulmonary disease

Neurotransmitters

Monoamine neurotransmitters (Fig. 1) have been used as markers of neurological health and function. Biologically, they are easily synthesized from simple amino acid reserves to yield the active signal transmitters. These active compounds include norepinephrine, epinephrine, dopamine, and serotonin, among others. Measurements of their metabolites are also very helpful in determining mechanism of action of certain therapeutic agents (reuptake inhibitors) and serving as markers themselves in various pathological states (DOPAC and DHPG in Parkinson’s Disease).

Traditionally, neurotransmitter analysis has been accomplished through the application of HPLC with electrochemical detection, due to the majority of analytes having very little absorbance activity for UV detection and their sensitivity to oxidation. However, these methods often suffered from poor sensitivity, interferences from complex samples, and extremely long analysis times [7]. More recent applications using LC-MS-MS have improved these areas of their analyses.

From an analytical perspective, neurotransmitters are very small and polar in nature. Their elution profiles on reverse phase HPLC shows them eluting early, under very weak chromatographic conditions. Extraction methods used for sample preparations for HPLC applications utilized alumina SPE, taking advantage of the polar diol functional groups of the catechol structure [8].

These characteristics make the analysis of this class of compounds difficult. For an LC-MS-MS application, a low molecular weight produces a high noise level, reducing sensitivity, and selectivity. Poor retention on reverse phase LC systems produces a poor desolvation condition, further limiting sensitivity.

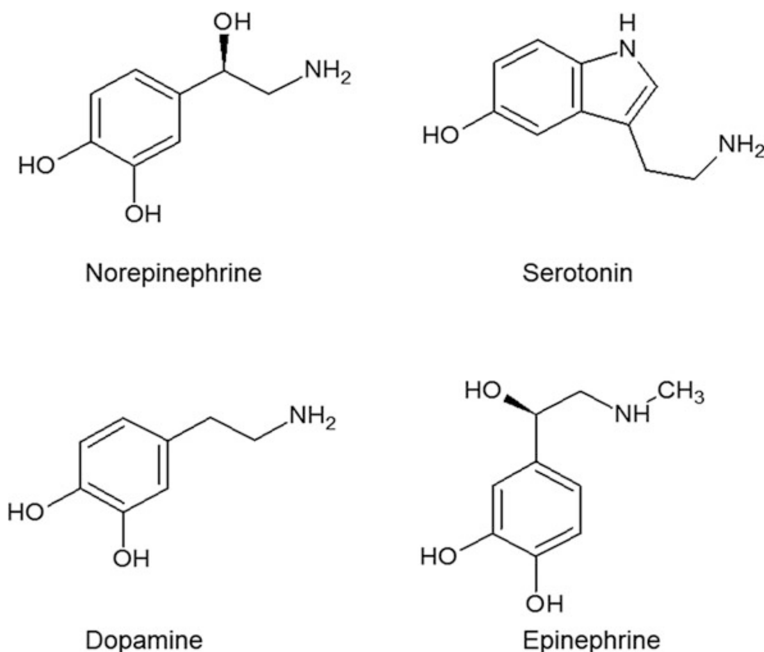

Fig. 1 Chemical structures of neurotransmitters (Norepinephrine, Serotonin, Dopamine, Epinephrine)

By using derivatization with dansyl chloride, issues with sensitivity can be improved [9]. The dansyl derivative leaves a larger molecule for chromatographic and mass spectrometric analyses. With better retention and lower noise from the higher ion transitions, sensitivity and selectivity are improved. In addition, several acid metabolites can also be analyzed under the same conditions that would otherwise require the opposite ionization polarity (negative ion electrospray). A similar solution to LC-MS-MS chromatographic retention and sensitivity through ethylation of epinephrine and norepinephrine after alumina extraction has been used. This allowed for a sensitive assay achieving detection limits to the low pg/mL level in plasma [10].

Derivatization of low molecular weight compounds or poorly ionized compounds can provide a powerful leverage to improve sensitivity by LC-MS-MS.

Vitamin D

Vitamin D analysis is a recent addition to LC-MS-MS techniques. Vitamin D levels in plasma are measured to assess the status of the individual, where low levels are associated with heart disease, diabetes, cancer, autoimmune disorders, and of course bone growth disorders [11].

Vitamin D originates from both endogenous (vitamin D_3) and dietary (vitamin D_2) sources. Both forms are quickly metabolized in the liver to 25-hydroxyvitamin D forms (25-(OH)D_3 and 25-(OH)D_2) which circulate with relatively long half-lives until they are converted to their active 1,25-dihydroxyvitamin D forms in the kidney. The active dihydroxy forms have a very transient existence and are present at extremely low levels. For these reasons, the concentrations of plasma 25-(OH)D_2 and 25-(OH)D_3 are considered the reference biomarkers for vitamin D nutritional status [11].

LC-MS-MS analysis of vitamin D provides all the expected advantages the hyphenated technique brings to other assays, which include improved sensitivity and selectivity, reduced processing and run times, and the ability to measure multiple analytes in a single analysis. However, limitations of the technique are demonstrated in the case of vitamin D. Common, nonspecific water loss product ion fragments make interferences from analogous compounds a potential problem. Also, an epimer of 25-(OH)D_3 is known to interfere on LC-MS-MS assays, while there is no such interference with immunoassays for vitamin D. These challenges are overcome with careful selection of mass spec settings and chromatographic conditions [11, 12].

For the analysis of 25-(OH)D_2 and 25-(OH)D_3 (Fig. 2), assays utilizing many different variables and setups have been successfully validated and used for clinical and drug development purposes. Electrospray as well as atmospheric pressure chemical ionization (APcI) sources have been used. Electrospray is known to be more susceptible to ion suppression events during analysis, and APcI used with vitamin D analysis has been shown to provide better precision of results presumably due to less suppression effects [12].

Product ion fragments using water loss have been commonly used for high signal response, but they have been subject to interferences not experienced with more substantial structural fragmentations. LC-MS-MS can easily distinguish between the precursor ions of 25-(OH)D_2 and 25-(OH)D_3 (413 and 401, respectively). However other hydrophobic lipids or structurally similar sterols have the potential to share these masses and a water loss transition is not unusual for this class of compounds. Listed transitions for 25-(OH)D_2 that have been used are 413 > 395, 413 > 377, 413 > 355, 413 > 337, 413 > 83, and for 25-(OH)D_3 are 401 > 365, 401 > 257, 401 > 159. During APcI ionization, the use of precursor masses with nominal water loss (395 for 25-(OH)D_2 and 383 for 25-(OH)D_3) is also common [12–14].

Sample preparation techniques used for vitamin D analysis include protein precipitation, liquid–liquid extraction, and solid phase extraction. The simpler extraction methods give shorter sample processing times, but often require more involved chromatographic separation and run time. One example of a method utilizing acetonitrile precipitation and filtering of the extract still required an LC system incorporating a switching valve and trapping column to effectively remove phospholipid interferences [13]. Liquid–liquid extraction techniques have been very effective for eliminating phospholipid interferences and allowing for shorter LC analysis times [12–14].

25-Hydroxyvitamin D2 25-Hydroxyvitamin D3

Fig. 2 Chemical structures of Vitamin D analytes (25-Hydroxyvitamin D2, 25-Hydroxyvitamin D3)

Steroids

Endogenous steroids are generally very lipophilic compounds that tend to ionize poorly, making them difficult to measure by LC-MS-MS, and yet, it is becoming the method of choice for steroid analysis. Advantages in analysis time, specificity, and performance at low concentrations make LC-MS-MS preferred over immunoassays.

The main challenges in steroid analysis by LC-MS-MS are poor ionization for sensitivity and susceptibility to interference from isobaric compounds. Recent methods have brought innovative approaches to deal with these problems.

In the analysis of estrone and estradiol (Fig. 3), very low detection limits are needed to quantify plasma samples in pre-pubescent females, men, and post-menopausal women. Methods that measure as low as 1 pg/mL are needed to detect even normal basal levels for the purpose of clinical analysis or drug development. Although both analytes are lipophilic, their chemical structures contain a phenolic hydroxyl group that can ionize in negative ion mode. This is a weakly acidic functional group, but the addition of ammonium fluoride in the mobile phase has been shown to improve the sensitivity of monitoring the native estrogen compounds presumably through improved ionization in negative ion mode [15, 16].

Liquid–liquid extraction appears to be the sample preparation of choice to give as clean an extract as possible [15]. Serial extraction is also sometimes employed to achieve improved recovery [16]. With such a low detection limit, further clean up with a trapping column prior to separation on the analytical LC column has also been used [16]. Dansyl derivatization of the phenolic hydroxyl group after extraction has been applied to estrone and estradiol in order to give an easily ionizable group to the structure [17]. This provides a way to improve the detection

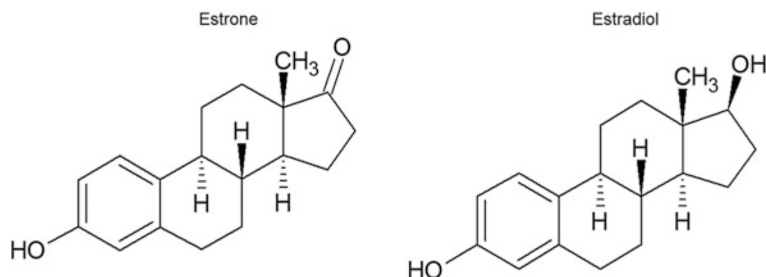

Fig. 3 Chemical structures of estrone and estradiol (Estrone, Estradiol)

sensitivity, but all applications seem to require a very selective chromatographic system in order to ensure required resolution between estrone and estradiol, as well as other potential isobaric interferences [15–17]. Other endogenous steroid methods follow similar schemes; extremely low detection limit, lipophilic extraction and concentration, derivatization (dansyl chloride, hydroxylamine), and a very selective chromatographic system often utilizing UHPLC [15, 18] to avoid isobaric interferences.

Key Term 3
UHPLC (**Ultra High Pressure/Performance Liquid Chromatography**): A chromatographic system similar to HPLC, but uses a smaller particle packing in the column (usually <2 microns). The smaller particles are closely packed and cause a high back pressure from the column, but peaks are sharper and resolution of peaks is obtained more rapidly.

Nicotine/Cotinine

Environmental tobacco smoke exposure is a concern from a clinical perspective due to its general effects on health (risk factor for cardiovascular diseases, cancers, and deaths) and in particular its impact on organ transplant. Smoking cessation efforts are considered an integral part of optimal solid organ transplant for tissue donors and recipient candidates. As an alternative to self-reporting, nicotine status is proposed as a biomarker for tobacco smoke exposure. Nicotine is one of the major components of tobacco which is quickly absorbed when inhaled and distributed in the blood. It is metabolized within a couple of hours to cotinine in the liver. Cotinine has a relatively long half-life in circulation (approximately 6–22 h), which gives it one advantage as a biomarker over short-lived candidates [19, 20].

Nicotine and cotinine (Fig. 4) are very small molecules, which makes their detection by LC-MS-MS as challenging as the neurotransmitters discussed earlier.

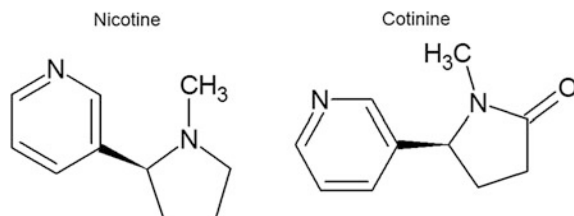

Fig. 4 Chemical structures of nicotine and cotinine (Nicotine, Cotinine)

Having low molecular weights allows for many interferences from the low end of the mass scale and provides background noise that limit sensitivity. Fortunately, the detection limits for the purpose of tobacco exposure are relatively high (1–2 ng/mL) which allows for the monitoring of the native underivatized structures. Common transitions used are 163 > 130 for nicotine and 177 > 80 for cotinine using electrospray positive ion mode. Chromatographic conditions are oriented toward reverse phase on long (>100 mm) HPLC columns under gradient elution. Sensitivity of the detector response to mobile phase components is not an issue, as mobile phase compositions tend toward traditional preparations; acetonitrile, methanol, and water, with and without formic acid and acetate salts [19–21].

Extractions for combined assays of nicotine and cotinine usually are performed using both ion exchange and reverse phase SPE. Due to the sensitivity requirements, the extracts are concentrated through evaporation prior to injection. Nicotine can be volatile compared to other small molecules, so in some cases an acid keeper (hydrochloric acid) is added before the drying step [19–21].

One of the more challenging aspects of the analysis of nicotine and its metabolites is finding clean matrix for use with calibration samples. Conceivably, identifying nonsmoking individuals should be sufficient to harvest nicotine-free plasma, but the presence of second-hand exposure to tobacco smoke, as well as potential dietary sources of nicotine are enough to produce responses that bias the lower level calibrators. For some, the use of pre-screened matrix from commercial sources is sufficient for use, but others found the need to scrub serum or purchase a nicotine-free synthetic matrix to have acceptably clean control blank samples [19–21].

When comparisons of different nicotine methods by LC-MS-MS have been made through analysis of common samples, good reproducibility of results has been reported [19]. This suggests the analysis of nicotine and cotinine is very reliable, even across different sample preparation and chromatographic conditions, once the sensitivity and endogenous presence challenges are under control. Correlation between nicotine response and cotinine response (corrected for sampling time) after dosing (nicotine patch) has been demonstrated to be good, confirming the utility of cotinine analysis as a nicotine status indicator [21].

The Future of Small Molecule Biomarker Analysis by LC/MS

As small molecule biomarkers become a greater part of the drug development process, the future holds several areas of expansion. One is the standardization of validation approaches for establishing a biomarker. In a regulated environment, specific recommendations are going to be made on how to approach the preparation of calibration curves for endogenous compounds and how to evaluate method validation parameters such as selectivity, limit of quantitation, and matrix effects. In addition, instrumentation capabilities are bound to improve. As technology improves in LC-MS-MS detectors, quantitation of very low level metabolites will be possible. This will open up the scope of possible biomarker candidates available for evaluation, expanding an already plentiful metabolome database [22]. Regardless of those advances, exploring the existing contents of the blood, CSF, and urine metabolomes brings more small molecule biomarker methods to light.

Key Term 4
Matrix Effect: The influence on analytical response (of the detector) by the presence of plasma, urine, or CSF (matrix) components in an extracted sample, as compared to a sample containing only solvent (no matrix). The presence of matrix can effect LC-MS ionization and it can also influence extraction recovery. This term is used to help explain inconsistencies of chromatographic peak responses from different samples during analysis.

Chapter Summary

- Small molecule biomarkers provide insight into metabolic pathways related to therapeutic treatment and can help explain the mechanism of action during drug development.
- LC-MS-MS method approaches parallel those for drug pharmacokinetic analyses; optimizations of detector, chromatographic system, and extraction are similar.
- Endogenous presence of small molecule biomarkers present a unique challenge for developing and validating an LC-MS-MS biomarker assay compared to excipient compounds.
- Other challenges and innovative solutions for small molecule biomarker assays:
  - *Sensitivity*—derivatization and optimized mobile phase composition are strategies that can improve signal response in the mass spectrometer.
  - *Ion suppression*—use of APcI can reduce ion suppression effects seen in electrospray ionization.

Selectivity—use of alternate phases (ion exchange, HILIC) besides reverse phase chromatography provides retention and separation based on different characteristics. UHPLC can increase throughput by resolving critical peak pairs in short time frames.

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