APPLICATION OF LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY IN CLINICAL LABORATORIES: A REVIEW

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ABSTRACT

Building an LC-MS/MS-based diagnostic test requires several technologies and skills. Journal publications seldom describe method development. Method developers may have to do substantial study to obtain test data. This research describes method development techniques. It also discusses technique development difficulties like assay calibration and quality control placement using literature examples. This review provides a comprehensive reference and encourages critical thinking about the research utilized to create a clinically relevant LC-MS/MS assay.

Key Words: *Tandem Mass Spectrometry, Liquid Chromatography, Method Development, Sample Preparation, Calibration, Internal Standards, Matrix Effects, Quality Control*

INTRODUCTION

Clinical testing for LC-MS/MS may be challenging. Chemical, physical, biological, and infrastructural issues must be addressed for an appropriate test result. Diagnostic MS testing is often done in-house. From calibration solutions to mobile phases, the lab prepares most testing materials. In development, the developer's ingenuity, experience, and resources limit the number of possible pathways to a final outcome. Method phases may be tailored to the lab's assets, process, and organization.

Combining toolkits typically creates a method. Tools may be bought, learned, or experimented with. Few publications detail method evolution. The author's experience generating hundreds of tests shows that method development trials fail more frequently than they succeed (for good cause). Publishable experiments are rare. A "final method" is detailed in a "method development" section, limiting vicarious learning. This study highlights sparse literature resources for clinical assay development, focusing on small-molecule measures. The lack of publications on complete MS peptide/protein measurement in clinical tests biases molecules < 1,000 Da. Many fundamental ideas apply to both small and giant molecules, although important discrepancies will be recognized. Matrix-assisted laser desorption-ionization-MS is used clinically, however most measurement methods require chromatographic separation before MS detection.

This research provides examples of LC-MS/MS utilized in clinical laboratories and clarifies assay development methods that are typically not included in previous publications. Method development considerations order sections. Even though this review is linear, the reader should return to relevant areas during method development to enhance procedure until the assay is validated.

ANALYTICAL MATERIALS

MS assays should begin with high-quality standard material, not equipment. Most clinical MS tests need a known amount of the measuring material. Acyl carnitines and urine organic acids, utilized in

newborn screening, need post-analysis data review and do not require a standard material. Analytical standards must be correctly supplied for testing. Our experience suggests getting at least two batches of standard material from separate manufacturers. This allows experiments to find weaknesses or evaluate data not in the certificate of analysis. A tidy standard's chemical manufacture and purification might cause concentration assignment bias. Errors may not be found until the validation procedure' method comparison phase or after the assay is launched [8]. When producing several comparable analytes from various solutions, pay special attention to metabolites and subtle changes [9]. Manufacturing or degradation byproducts may bias measurand concentrations.

In certain cases, higher-order metrology-connected certified reference material assigns value and error. The International Federation of Clinical Chemistry, National Institutes of Science and Technology, Joint Committee for Traceability in Laboratory Medicine, National Measurement Institute Australia, National Metrology Institute of Japan, and Korea Research Institute for Science and Standards offer compounds [10]. These may appear in vendor catalogs. Quantitative nuclear magnetic resonance has increased access to traceable materials with well-characterized concentrations and improved calibration-related metrology.

An MS test requires an analytical standard to assess medication in patient samples. Without a pure material, experimental designs have a lot of assumptions and few variables. Spike and recovery evaluations are easy with a clean drug. Alternative methods generally need extra investigation before testing patient samples.

INTERNAL STANDARD (IS) SELECTION

MS clinical analysis needs IS. MS technology's rather robust response functions at low concentrations, apparent specificity due to collisional dissociation, and high resolution are vital, but the capacity to correct for all analytical processes with a true physicochemical mimic is crucial. Endogenous analytes need IS investigations. Without endogenous analyte bias, IS recovery may measure assay efficiency in patient samples [13]. Time-course studies may assess endogenous protein binding or adsorptive loss [14, 15]. Equilibration time affects IS normalization and analyte recovery. Method development should determine patient sample IS equilibration time and conditions.

Proper IS labeling should resolve high-level analyte isotopic contribution. Fortunately, many stable, isotopically labeled ISs may exist. IS selection relies on deuterium isotope effect (for deuterium-labeled species), cost, availability, and product ion creation reliability.

Journals seldom clarify IS concentration requirements for tests. Cost, pipetting accuracy, solubility, stability, assay compatibility, unlabeled analyte, and analytical precision are factors. The lowest IS concentration (cost) should provide the least noise and incorrect integration (analytical accuracy) within the detector's linear range. Titrations, repeat injections, and detection accuracy are evident development experiments. Most journals don't clarify fundamental experiments. "Evidence-based medicine" demands fact-based reasoning.

MS DEVELOPMENT

After gathering analytical materials, procedure development might begin.

Establishing intermediate MS parameters like collision energy and precursor/product ions is sensible. The source condition should be appropriate for low-flow infusion but not for the final procedure. After including source performance parameters like LC flow rate and solvent composition, substantial refinement is appropriate.

MS seldom deviates from the expected protonated precursor. In-source dissociation may lose water. In-source dissociation depends on electronics and temperature. The initial infusion should evaluate these. Some analytes release adducts instead of the weakly deprotonated precursor ion. Depending on the molecule(s), lithium, sodium, ammonium, and other elements may generate common adducts.

The infusion solution's possible adducting species may create new precursor ions.

CHROMATOGRAPHY DEVELOPMENT

Chromatographic development concerns for diverse applications have been addressed. Clinical laboratory testing is not considered in these rules. Large sample sizes or masses provide bigger response functions with a given analyte load for active pharmacological component assessment. Most clinical tests are impractical. Newborn urine is sparse and hard to collect, despite appearances. Clinical blood draining is no longer recommended. Samples are few. MS seldom allows greater analyte reactions. Increase the source's ion yield. Avoid larger patient sample sizes.

Chromatographic bioanalysis for clinical trials may not be adequate to measure endogenous substances in uncontrolled test populations. Laboratory sample types may only be observed in trials. Diagnostic opaque yellow plasma from severe lipemia or near-neon orange icteric serum are common. Bioanalysis recommendations should be treated with caution, even if they are most directly connected to diagnostics.

SAMPLE EXTRACTION

Sample preparation has various types for different purposes. Start by finding the instrument's lowest exact response to the reference interval. Consider a 1 ng/mL LLOQ test. SPE or liquid-liquid extraction is unnecessary if the assay can detect 1 pg/mL with high signal-to-noise ratio and suitable solvents and separation. These preparative methods provide selectivity the LC system cannot. High-resolution, near-automated LC. Selective sample extraction may increase specificity before MS detection when prepared for the target compound(s), however LC is typically better.

After deciding whether to concentrate or dilute extraction, analyze the lab's organization and equipment. A 24-position SPE vacuum manifold lab that runs 1,000 tube-based SPE samples per day will need extra vacuum ports and manifolds. Affordable positive-pressure 96-well plate manifolds. During method development, quality science in production or industry may take precedence over research goals.

Extraction development benefits from summarizing various experiment concepts. Matrix-based tidy solution research is crucial. Many sample matrices are preserved. in citrated plasma. Absolute analyte response, IS recovery, peak shape variations, retention time drifts after numerous injections, and visible debris or other macro-confounders in ex-tracts are relevant assessment criteria. Method development involves diligent experimentation with diminishing data.

CALIBRATION MATRIX

Early method development requires no human matrix. Assays may be made using simple commercial components. However, add the sample matrix immediately. This correctly assesses calibration materials. CLSI's calibration test item structure. A calibration matrix should be interchangeable, accessible, and analytically similar to the matrix of interest. Many LC-MS/MS studies struggle to choose the best patients for a particular analyte concentration. MS typically analyzes endogenous targets utilizing analyte-depleted matrix, synthetic matrix, or solvent-based methods. These materials may be stabilized and adsorbed by preservatives and binding partners. External analyte matrices are commercial or compound-free. Analysis of fast-metabolizing substances like cocaine in whole blood requires precise collection, transit, and storage conditions.

Assay quality depends on calibration matrix. LC-MS/MS calibration only requires accurate preparation. In the initial stage of a technique, adding an IS to sample aliquots, accuracy is important. IS and sample aliquots must match calibration standard ratio. IS and sample control absolute recovery errors during sample preparation, injection, and ionization. Only calibration standards need accurate preparation.

Prudence is needed when comparing the calibration matrix and samples. Due to lipophilic component removal, charcoal-stripped serum behaves differently from human serum. Dialyzed calibration matrices may contain less endogenous material. Deviations of the calibration matrix from the human matrix may affect adsorptive loss, matrix effects during sample preparation, IS equilibration duration, and ion-ization suppression in calibrators and specimens.

CALIBRATION CURVE CONCENTRATIONS

Calibration curve concentrations aren't strictly regulated. The FDA and EMEA advise against MS diagnostics. The EMEA and US FDA need six distinct standards and 15% back-calculated accuracy for non-LLOQ levels and 20% for the LLOQ. Since they were designed for clinical trials of new pharmacological substances, these recommendations may not be suitable for diagnostic testing.

These instructions mention several parts. First, the calibration range should always include two calibrated standards, one at LLOQ and one at ULOQ. Extrapolating values below the LLOQ (without a concentration factor) or above the ULOQ (without a dilution) is not recommended. The prolonged low-end imprecision of LC-MS/MS analysis acknowledges its heteroscedastic nature. Such guidance disagrees with the CLSI EP17-A2 procedures for setting lower and upper limits of measurement intervals (LLMI and ULMI, respectively). Limit of blank and limit of detection methods usually need numbers lower than the lowest calibrator, which would be more inaccurate. The EMEA and US FDA publications advocate LC-MS/MS, although the CLSI advice guides include a number of test methodologies, including LC-UV detection, nephelometry, turbidity, and PCR. The suggestions are preferred because they examine the platform's strengths and drawbacks. Thus, the ULOQ and LLOQ match the LLMI and ULMI. LC-MS/MS calibration curve points should also account for non-linear ionization and detection. The dynamic range of modern MS systems is not infinite. Ionization and detection non-

linearity may fluctuate over time on the same instrument and vary by vendor and type. Carryover may restrict orders of magnitude. LC-MS/MS seldom has a working range exceeding 2,000-fold LLMI-ULMI difference.

UNDERSTANDING MATRIX EFFECTS

matrix effects in LC-MS/MS. The patient sample's ionization is stimulated or inhibited by the solvent matrix. alterations in measured response caused by a matrix during chromatography or sample preparation. examining the impacts of the ionization matrix. It is possible to lessen matrix effects by altering the extraction procedure, switching from electrospray to atmospheric pressure chemical or electron ionization, adding an in-line trapping column for lipidaceous suppressors, or utilizing preparative depletion. Everything is negative. Some molecules may not ionize enough to signal. Depletion of phospholipids may eliminate target compounds. The experimental strategy should manage unexpected results and matrix effects.

Ionization matrix effects are best resolved by increasing chromatographic resolution. It is simple to change the strong solvent amount after three vacant volumes. Use isocratic stages to resolve suppressed analyte species before the gradient. Once the response function has adjusted the solvent conditions, changing pH to alter resolution is not recommended. preparation of orthogonal samples or column screening.

Suppression or augmentation of ionization is essential. We've heard 200% amplification, 80% suppression, and a maximum solvent-to-matrix shift of 15%. Expectations are established by the lab's SOP. If the lab regularly accepts samples with an IS recovery of 50%–150% in comparison to the calibrators/QCs in the same batch, method development is modest. The development of a method differs from routine analysis, and stricter allowance criteria might reduce the robustness of an operational assay.

Matrix effects must be taken into consideration during sample preparation and chromatography. Effects of the IS binding partners and the analyte impede sample preparation. Binding may result in differences in the recovery of the analyte and the IS [16, 17]. Vulnerable substances include non-specific agents and analytes with strong binding partners. Time-course analyses look at the sample preparation process. Depending on stability, the exogenous measurement analyte may be concentrated and left to equilibrate with the matrix for hours or days. Endogenous sample data are kept. IS-applied reproductions that were withdrawn. Area ratios are repeated over time after assessing the analyte's absolute response and IS. Temperature changes in the lab may hasten the equilibration of the IS-analyte. Never assume that a technique for extracting a bound analyte entirely releases it or that IS has achieved equilibrium. Numerous illnesses may alter the non-specific binding of patient samples. Before experimentally proving that the extraction process may release any bound fraction and/or achieve equilibrium with the IS, a number of assumptions must be established.

OPTIMIZATION

The optimization of the LC-MS/MS assay is hampered by several conflicting factors. Enhance an assay for drug metabolites. Consider, for philosophic purposes, that the drug has a weak ionization cross-section and a short half-life, whereas the

metabolite has a large one and a long half-life. Medicine may need to be studied at concentrations 100 times lower than the experimental metabolite. When a drug is optimized, the signal is maximized, however when a metabolite is optimized, it may be necessary to use less-than-ideal settings in order to avoid source saturation or detector blindness and identify both molecules from a single preparation or injection.

MS optimization entails more than just "achieving the highest signal," notwithstanding the goal. Enhance productivity, possible error rates, raw data noise, robustness, and throughput. Because of the many instrument kinds and goals, literature cannot provide accurate suggestions. Writing recycles.

As mentioned previously, LC optimization, sample preparation, and MS parameter optimization. Change the temperature, the probe's voltage and location, the collision and ion optics energies, and the gas pressures and flow rates. Techniques for single and multiple variables are provided. Both techniques impact the analyte and noise. Be careful, the components of the MS analysis are interrelated. The ideal collision energy vary depending on the collision cell pressure. Diverse contacts are required for optimization research.

CONCLUSION

In our discussion of LC-MS/MS studies, we covered the following topics: sequestering test materials, establishing MS parameters, chromatographic separation, sample preparation, calibration and QC materials/concentrations, and matrix effects. A method development check list that roughly corresponds to assay production is shown in Table 4. Some topics may not be covered in this study or the literature. The most fulfilling aspect of science may be the discovery and resolution of such issues. After these problems are resolved, patient testing may be improved by intelligent experimental design informed by all LC-MS/MS system parameters.

Neither pre-validation nor validation were used in our study. We also put off talking about how to set up and run a production assay for a very long time. These crucial characteristics for clinical lab LC-MS/MS will be covered in the second installment of this review series.

The canvas of LC-MS/MS experiments is not particularly constrained. Thus, scientific innovation and—more importantly—the lack of data supporting the use of such tests on patient samples limit the ability to develop very efficient and high-quality diagnoses. The papers presented here just skim the surface of one method for producing high-quality work; the development scientists are left to handle the bulk of it. Finally, "Good luck and keep working hard."

REFERENCES

1. Benton SC, Tetteh GK, Needham SJ, Mücke J, Sheppard L, Alderson S, et al. Evaluation of the 25-hydroxy vitamin D assay on a fully automat- ed liquid chromatography mass spectrometry system, the Thermo Sci- entific Cascadion SM Clinical Analyzer with the Cascadion 25-hydroxy vitamin D assay in a routine clinical laboratory. Clin Chem Lab Med 2020;58:1010-7.

2. Metz TF, Mechtler TP, Merk M, Gottschalk A, Lukačin R, Herkner KR, et al. Evaluation of a novel, commercially available mass spectrometry kit for newborn screening including succinylacetone without hydrazine. Clin Chim Acta 2012;413:1259-64.

3. Polledri E, Mercadante R, Ferraris Fusarini C, Maiavacca R, Fustinoni S. Immunosuppressive drugs in whole blood: validation of a commercially available liquid chromatography/tandem mass spectrometry kit and comparison with immunochemical assays. Rapid Commun Mass Spec- trom 2017;31:1111-20.

4. Duncan MW, Nedelkov D, Walsh R, Hattan SJ. Applications of MALDI mass spectrometry in clinical chemistry. Clin Chem 2016;62:134-43.
5. Croxatto A, Prod'hom G, Greub G. Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. FEMS Microbiol Rev 2012;36:380-407.
6. Millington DS, Norwood DL, Kodo N, Roe CR, Inoue F. Application of fast atom bombardment with tandem mass spectrometry and liquid chromatography/mass spectrometry to the analysis of acylcarnitines in human urine, blood, and tissue. Anal Biochem 1989;180:331-9.
7. Jones PM and Bennett MJ. Urine organic acid analysis for inherited metabolic disease using gas chromatography-mass spectrometry. In: Garg U., Hammett-Stabler C. (eds) Clinical Applications of Mass Spec- trometry. Methods in Molecular Biology (Methods and Protocols), vol 603. Humana Press, 2010:423-31.
8. Singh RJ, Grebe SK, Yue B, Rockwood AL, Cramer JC, Gombos Z, et al. Precisely wrong? Urinary fractionated metanephrines and peer-based laboratory proficiency testing. Clin Chem 2005;51:472-3; dis- cussion 473-4.
9. Hanari N, Itoh N, Ishikawa K, Yarita T, Numata M. Variation in concen- tration of perfluorooctanoic acid in methanol solutions during storage. Chemosphere 2014;94:116-20.
10. Wise SA. What is novel about certified reference materials? Anal Bio- anal Chem 2018;410:2045-9.
11. Choi K. Purity assessment using quantitative NMR: establishment of SI traceability in organic analysis. J Korean Magn Reson Soc 2020;24:30- 7.
12. Malz F and Jancke H. Validation of quantitative NMR. J Pharm Biomed Anal 2005;38:813-23.
13. Grant RP. Everything you wanted to know about internal standards but were too afraid to ask. Part 1: What, why, and how. https://www.msacl.org/presenter_slides/2019_US_grantr_74175_slides.pdf (Updated on Nov, 2020).
14. Shuford CM, Walters JJ, Holland PM, Sreenivasan U, Askari N, Ray K, et al. Absolute protein quantification by mass spectrometry: not as simple as advertised. Anal Chem 2017;89:7406-15.
15. Leinenbach A, Pannee J, Dülffer T, Huber A, Bittner T, Andreasson U, et al. Mass spectrometry-based candidate reference measurement procedure for quantification of amyloid-β in cerebrospinal fluid. Clin Chem 2014;60:987-94.
16. Tai SS and Welch MJ. Development and evaluation of a reference measurement procedure for the determination of estradiol-17β in hu- man serum using isotope-dilution liquid chromatography-tandem mass spectrometry. Anal Chem 2005 1;77:6359-63.
17. Tai SS and Welch MJ. Development and evaluation of a candidate ref- erence method for the determination of total cortisol in human serum using isotope dilution liquid chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry. Anal Chem 2004; 76:1008-14.
18. Lindegardh N, Annerberg A, White NJ, Day NP. Development and vali- dation of a liquid chromatographic-tandem mass spectrometric method for determination of piperaquine in plasma: stable isotope labeled internal standard does not always compensate for matrix effects. J Chromatogr B Analyt Technol Biomed Life Sci 2008;862:227-36.
19. Berg T and Strand DH. 13C labelled internal standards-a solution to minimize ion suppression effects in liquid chromatography-tandem mass spectrometry analyses of drugs in biological samples? J Chro- matogr A 2011; 1218:9366-74.
20. Davison AS, Milan AM, Dutton JJ. Potential problems with using deu- terated internal standards for liquid chromatography-tandem mass spectrometry. Ann Clin Biochem 2013;50:274.
21. Eppendorf AG. How to pipette small volumes with handheld manual pipettes. https://handling-solutions.eppendorf.com/liquid-handling/pipetting-facts/small-volumes/detailview/news/how-to-pipette-small-vol- umes-with-handheld-manual-pipettes (Updated on Sept 2020).
22. Liigand J, Laaniste A, Kruve A. pH effects on electrospray ionization ef- ficiency. J Am Soc Mass Spectrom 2017;28:461-9.
23. Rauha JP, Vuorela H, Kostiainen R. Effect of eluent on the ionization efficiency of flavonoids by ion spray, atmospheric pressure chemical ionization, and atmospheric pressure photoionization mass spectrome- try. J Mass Spectrom 2001;36:1269-80.
24. Deventer K, Pozo OJ, van Eenoo P, Delbeke FT. Development and vali- dation of an LC-MS/MS method for the quantification of ephedrines in urine. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877:369- 74.
25. Eyles D, Anderson C, Ko P, Jones A, Thomas A, Burne T, et al. A sensi- tive LC/MS/MS assay of 25OH vitamin D3 and 25OH vitamin D2 in dried blood spots. Clin Chim Acta 2009;403:145-51.
26. Ferreiro-Vera C, Ribeiro JP, Mata-Granados

JM, Priego-Capote F, Luque de Castro MD. Standard operation protocol for analysis of lipid hydro- peroxides in human serum using a fully automated method based on solid-phase extraction and liquid chromatography-mass spectrometry in selected reaction monitoring. J Chromatogr A 2011;1218:6720-6.

27. *Kuhn J, Götting C, Kleesiek K. Sample cleanup-free determination of mycophenolic acid and its glucuronide in serum and plasma using the novel technology of ultra-performance liquid chromatography-electro- spray ionization tandem mass spectrometry. Talanta 2010;80:1894-8.*
28. *Bittersohl H, Schniedewind B, Christians U, Luppa PB. A simple and highly sensitive on-line column extraction liquid chromatography-tan- dem mass spectrometry method for the determination of protein-un- bound tacrolimus in human plasma samples. J Chromatogr A 2018; 1547:45-52.*
29. *Rappold BA. Mass spectrometry selectivity, specifically. Clin Chem 2016;62:154-6.*
30. *Bunch DR, McShane AJ, Wang S. Investigation of transition ion ratio variation for liquid chromatography-tandem mass spectrometry: a case study approach. Clin Chim Acta 2018;486:205-8.*