Special Considerations for Liquid Chromatography–Tandem Mass Spectrometry Method Development

Brian A. Rappold

KEYWORDS

• Method development • Liquid chromatography • Mass spectrometry • Endogenous • Internal standards • Validation

KEY POINTS

- Optimization of liquid chromatography–tandem mass spectrometry methods during development is iterative through the developmental pipeline.
- Isotopically labeled internal standards provide near perfect surrogates for endogenous analytes when testing true human matrices.
- Modulation of chromatographic separations can elucidate nonspecificity of detection and should be used in method development.
- Aspects of method development that are outside commonplace evaluations include assessments of stoichiometry in pathologic states for derivatized workflows, area ratio monitoring for precision evaluations, and assessment of multiple sources of calibration material.

Method development for liquid chromatography–tandem mass spectrometry (LC-MS/MS) assays involves numerous components, including the varieties of sample preparation and LC as well as the advantages and limitations of MS. It is not uncommon that a simplistic view is provided with regard to method development, particularly in publications, which undervalues the complexity of method development. For example, sample preparation is generally divided into 4 classes, all of which may be used in the same workflow, those being protein precipitation (or simple dilution), liquid-liquid extraction, solid-phase extraction, and analyte modification (eg, derivatization or proteolysis). Yet within each of those procedures is a host of possibilities and variables: solid-phase extraction might use ion exchange, hydrophobic or hydrophilic mechanisms, or a combination. And each mechanism might use a variety of solvents, stationary phase ligands, stationary phase masses, wash steps, stationary phase-drying steps, elution steps, and evaporation/reconstitution steps, with each iteration possibly playing an essential role in the viability of the assay to be applied to the analysis of human specimens for diagnostic purposes. The landscape for method development is extensive.

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Mass Spectrometry Laboratory Corporation of America, 1904 TW Alexander Drive, Durham, NC 27703, USA
E-mail address: rappolb@labcorp.com

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Unfortunately, many of the details regarding method development are not included in descriptions found in most journals. Seemingly, method development occurs with only a plurality of positive outcomes. Articles often report final results for method development, not the actual method development itself. Take for example, a recent reference method procedure for amyloid beta 1 to 42, which uses ion exchange solid-phase extraction.[1] The investigators note adding additional washes to a previously published procedure, including a 4% aqueous phosphoric acid wash and increasing elution volumes. It is unclear what benefit a strong acid wash and changes in elution volume yielded, and perhaps more importantly, how those modifications were scientifically determined to be appropriate. That is not to say that the assay is unsuitable, but it does point out the absence of a description of method development. Rather, it includes a description of the final method. This might be because many experimental results in method development are negative and there is a bias toward not publishing negative outcomes.[2]

MASS SPECTROMETRY

Determination of MS parameters is generally the first step in method development. Identification of initial parameters, such as precursor ions, product ions, and source conditions must be established before further assay interrogation. Simply, data from chromatographic and sample preparation are fallible when detection is not performed in a consistent and high-quality manner. Many assays use multiple precursor-product transitions of a single analyte in the assessment of ion ratios to provide additional confidence in results. At the onset of development, the purity of a peak in human specimens (selectivity) has not been determined. As such, all available transitions should be maintained until such point that a precursor/production pair can be determined to be useful as a quantitative ion, a qualifying ion, or is excluded as being susceptible to interferences. The choice of removing a transition in method development should be entirely driven by specificity of analysis; dose-response functions for a particular ion transition can be modulated by ionization cross-section, dissociation efficiency, sample preparation, sample volume used and load of the sample in to the instrument. Given the ability of current-day MSs to scan quickly across multiple transitions, development of assays should move forward with all available possibilities until data definitively prove the quality of a particular MS/MS transition.

Notably, however, there are certain transitions that must be used with care.[3,4] When using MS/MS transitions that are either ubiquitous or facile, ensuring reproducibility and specificity in many authentic human specimens should be extensively evaluated in prevalidation and well proven in validation.

Reproducibility of a particular transition is quickly assessed by an evaluation of analyte to internal standard peak area ratios in early method development. In mass spectrometric assays with isotopically labeled internal standards (IS), it is assumed that most analytical variation is normalized by the IS. Inaccuracy should then be attributable only to the aliquoting of sample and the addition of IS. In practice, however, there may be some inaccuracy in the detection of compounds related to in-source variation or dissociation differences. **Table 1** shows exemplar data from the performance of this experiment for oxycodone using 2 transitions for the analyte and 2 transitions for the internal standard. Here, the analyte and internal standard are aliquoted to a single vial in neat solvent at a concentration intended to yield a moderate response in the mass spectrometer (high enough to provide confidence in the signal, low enough to avoid source and detector nonlinearity). The solution is injected in replicates (20 in this example) to determine the imprecision of analyte peak area to