

Immunosuppressant Drugs

Measurement by Tandem Mass Spectrometry

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Surgeons performed the first successful organ transplant of a kidney in 1954. Since then, organ transplantation has become a potentially lifesaving treatment for end-stage diseases of the kidney, liver, heart, lung, and pancreas. In 2005, the United Network for Organ Sharing (UNOS) reported that surgeons performed 28,106 transplants in the U.S. The need for organs far exceeds the number of donated organs, however, and currently UNOS has a waiting list of over 91,000 patients in need of a solid organ transplant. For patients who do receive a donated organ, immunosuppressant drug therapy and concentration monitoring of immunosuppressants play a critical role in the success of the transplant.

In the U.S., the four most common immunosuppressant drugs taken by transplant patients are cyclosporine A, rapamycin (sirolimus), FK506 (tacrolimus), and mycophenolic acid. Blood concentrations of each of these drugs must be monitored in order to avoid serious toxicities associated with concentrations above the therapeutic range. Furthermore, absorption and metabolism of immunosuppressants is quite variable among patients and within individual patients (1). Consequently, laboratory monitoring of immunosuppressant drugs is especially important during the initiation of drug therapy, and for most patients, some schedule of therapeutic drug monitoring will be maintained for life.

Methods for measuring immunosuppressant drug concentrations in blood include immunoassay, high-performance liquid chromatography (HPLC), and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (2,3). Among

development of high-level technical expertise on the part of our Special Chemistry staff. In this article, we describe our experience in setting up and performing immunosuppressant measurements using LC-MS/MS as well as technical and business considerations for

Ions formed in the mass spectrometer's electrospray source obtain momentum along the quadrupole's main axis by the presence of charged plates at the entry point. From among a collection of entering ions, the quadrupole can control with high resolution which ions are allowed to pass through based on their mass/charge (m/z) ratio.

Tandem mass spectrometers have three quadrupoles (Figure 1B). The first quadrupole, designated Q1, is a mass filtering quadrupole as described above. The second quadrupole, Q2, acts as a collision-activated dissociation (CAD) chamber, in which the presence of a CAD gas (e.g., nitrogen) can cause fragmentation of the ion that has passed through Q1. The third quadrupole (Q3) filters a particular ion from among those exiting Q2 and allows it to travel to the detector. Q1 and Q3 therefore act "in tandem" as mass filters. The selection of a Q1/Q3 ion pair ("parent" and "daughter" ions) can make the quantitation of Q3 ions highly specific for the compound of interest. The advantage of the tandem configuration over that of a single quadrupole is that amounts of specific compounds can be quantified from among a complex mixture of compounds—potentially even without prior chromatographic separation. The tandem mass selector configuration (MS/MS, accounting for two quadrupole mass selectors) is also known as a triple-quadrupole (accounting for the CAD chamber that is the middle quadrupole). The term LC-MS/MS is used when the MS/MS is coupled to a liquid chromatography system (LC) that introduces the sample into the ionizing source of the mass spectrometer.



these methods, LC-MS/MS is increasingly being used by clinical laboratories for routine measurement of immunosuppressants. In fact, the number of laboratories using MS in the College of American Pathologists (CAP) proficiency testing surveys for immunosuppressants has more than doubled—from 16 to 40—in just the past two years.

Three years ago, when we initiated efforts to measure immunosuppressants by tandem MS, this technology was entirely new ground for our clinical laboratory, and it required the

implementing and routinely using this advanced technology in the clinical laboratory.

A Primer: Quadrupole and MS/MS

In one form or another, mass spectrometry has been used for chemical analysis for more than 100 years, and the quadrupole mass filter was first described more than 50 years ago. The quadrupole consists of four conducting metal rods that bear opposite charges at alternating positions (Figure 1A). Electronic circuits control the rod voltages.

LC-MS/MS: A Scheme for Simultaneous Drug Measurement

Numerous methods for measuring immunosuppressant drugs have been published. Our laboratory developed an LC-MS/MS procedure based on the work of Volosov et al. (3) that simultaneously measures three of

Quadrupole mass filter

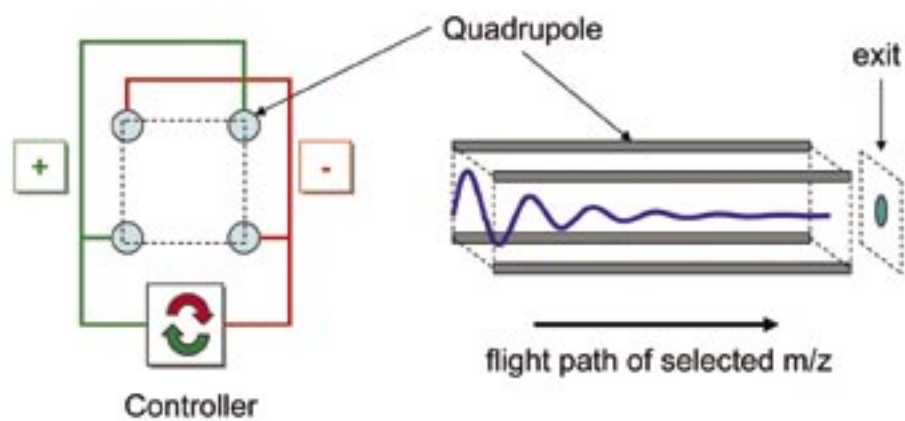


Figure 1A. **Principle of the quadrupole as a mass selector.** The quadrupole consists of four metal rods. At any instant, adjacent rods have voltages opposite in sign; a controller in turn controls the sign and magnitude of voltages as a function of time. The effect of this alternating field is to allow only ions with a fixed mass/charge ratio (m/z) to have a stable trajectory that can pass to the major-axis exit (and eventually to the detector) as shown. All other ions have unstable trajectories and exit the mass spectrometer through the vacuum exhaust.

Tandem mass selectors

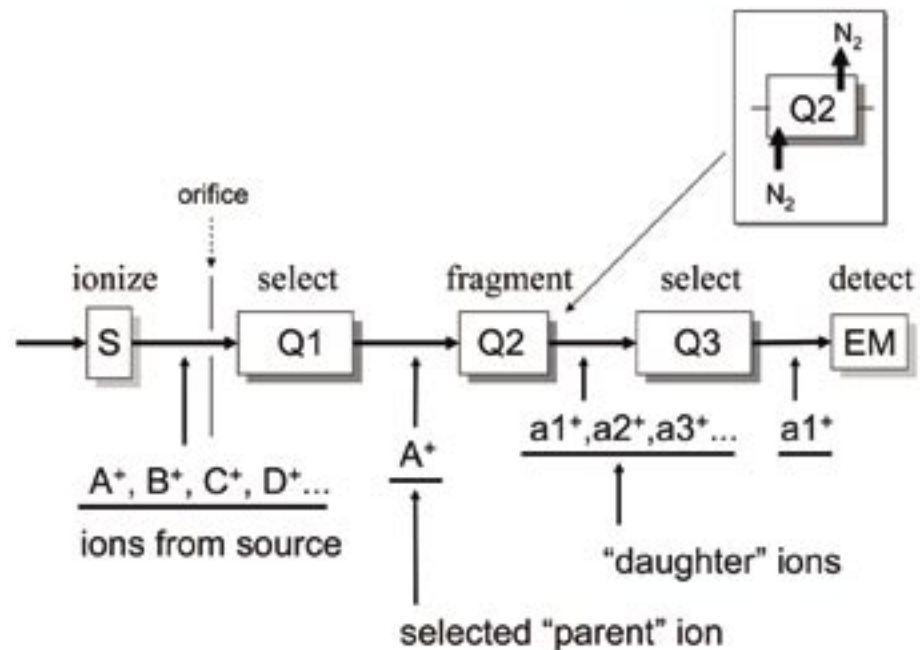


Figure 1B. **Schematic of a tandem mass spectrometer.** Ions from the atmospheric pressure source (S) enter the high vacuum mass spectrometer through an orifice and are filtered for specific masses in Q1 and Q3. Q2 is the collision-activated dissociation (CAD) chamber which includes flow of a CAD gas such as nitrogen or argon (inset). Not shown are additional quadrupoles used for beam focusing, and various fixed voltage plates used to induce or alter momentum along the major axis. The detector is an electron multiplier (EM) device.

the common immunosuppressants—cyclosporine, tacrolimus, and rapamycin. We take whole blood samples supplemented with internal standard, treat them to precipitate protein, and extract the drugs into an organic phase. An LC system is used to introduce an aliquot of the organic phase extract into the MS/MS via an autosampler (Figure 2). A small C-18 column, or “guard” column, holds the drugs for a wash step before elution of the drugs from the column by a two-component mobile phase gradient.

Key to the protocol is operation of the MS/MS in “multiple reaction monitoring mode” (MRM). We devised an MRM table using the instrument’s software to specify the measurement of a list of parent and daughter (Q1/Q3) ion pairs in sequence (Table 1). The complete MRM table includes analyte-specific instrument parameters, such as various voltage settings, that are empiri-

cally obtained to optimize the sensitivity of the MS/MS measurement for each analyte. At any instant, the mass spectrometer operates using only one set of parameters on the list, measuring a particular Q1/Q3 pair. This measurement occurs only for a relatively short, specified period of time (e.g., 50 msec) before the MS/MS proceeds down the MRM list to the next entry and its set of conditions. Upon completion of the list, the MS/MS begins again at the beginning of the list, so that the same list of operating/measurement conditions is continuously repeated. Therefore, the data collected for any one analyte in this multi-analyte “simultaneous” assay are in fact a set of discontinuous measurements.

In a six-analyte MRM table, the total measurement time devoted to any one analyte is somewhat less than one-sixth of the total MS/MS run time for each sample, tak-

ing into account the pause and reset time between each measurement interval. The discontinuous data are sufficient, however, to define a peak for each of the analytes (Figure 3). For operations using a bolus injection of sample, the number of entries in the MRM table is limited only by whether the resolution of the data is sufficient to accurately define each analyte’s peak shape and integral. The ratio of the analyte integral to that for its internal standard is then used to obtain the original drug concentration by comparison to a standard curve (Figure 3). Note that a quantitative assay could in principle be obtained without an internal standard; however, the internal standard can help to correct for variable losses and sample-dependent

ion suppression and matrix effects (4).

A promising area of the application of MS/MS for measurement of immunosuppressants is the ability to provide analysis of metabolite concentrations in addition to the concentration of the parent compound, as was recently demonstrated for mycophenolic acid (5). As metabolite profiles are expected to be clinically meaningful (6), a future possibility is that part of the standard of practice for immunosuppressant monitoring will be to provide a metabolite profile along with each measurement of the parent drug.

Technical Considerations

Prior to use of the LC-MS/MS assay, the lab must optimize the MS/MS operating

Table 1

Sample MRM Table for Combined Immunosuppressant Assays

Entry #	Analyte	Q1* (m/z)	Q3 (m/z)
1	cyclosporine A	1220.0	1202.7
2	Everolimus	975.5	908.5
3	Sirolimus	931.6	864.5
4	Tacrolimus	821.6	768.3
5	ascomycin (internal standard)	809.4	756.4
6	cyclosporine D (internal standard)	1234.0	1216.5

* In all cases, Q1 species represent an ammonium adduct ($[M.NH_4]^+$) of the analyte (M) that is formed in the ion source by combination with ammonium acetate present in the LC mobile phase.

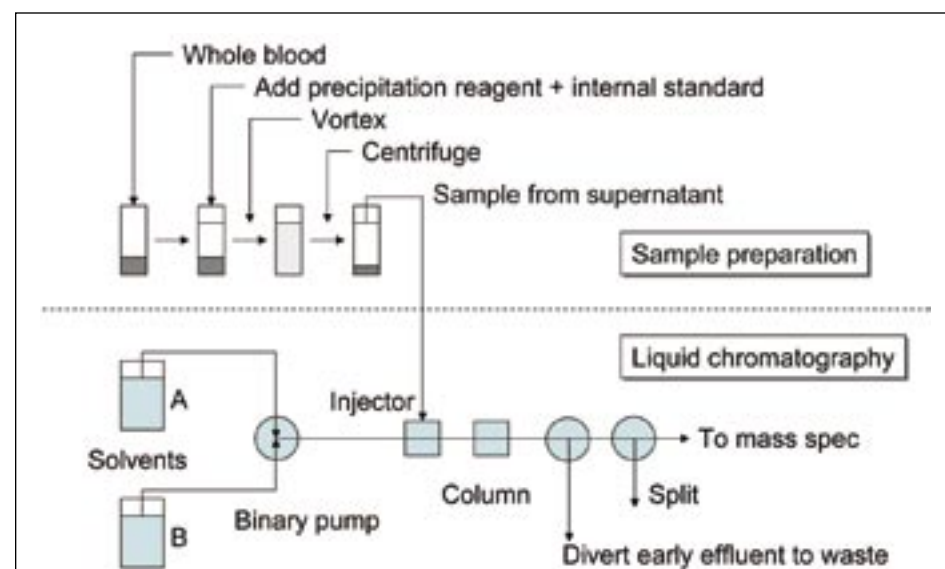


Figure 2. **Schematic flowchart of sample preparation and chromatography for immunosuppressants.** The time control of the LC portion of the procedure includes that of a post-column switch valve that can send effluent to waste or to the mass spectrometer. Additionally, there is a split of the LC output prior to entry to the source in order to limit the source flow rate.

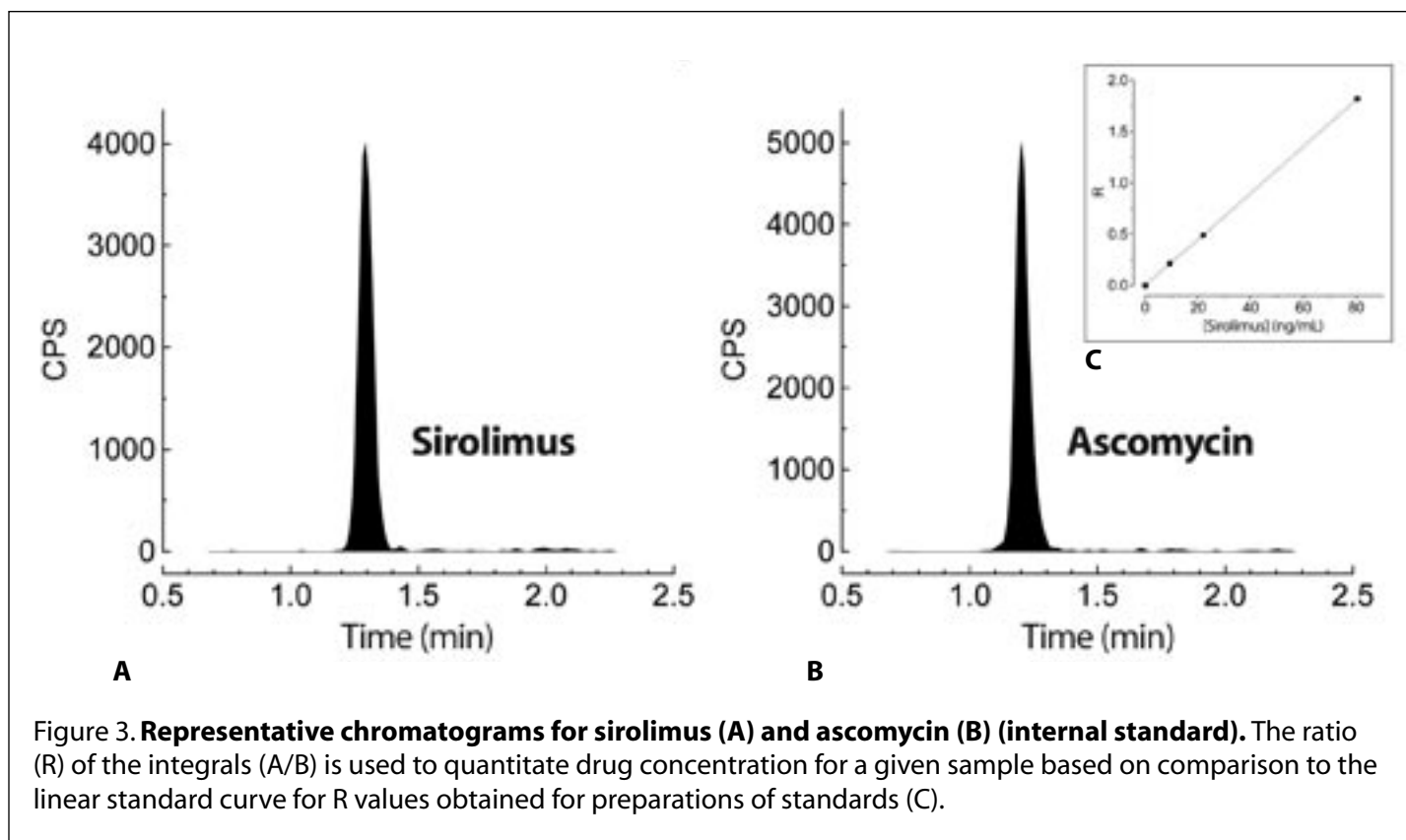


Figure 3. Representative chromatograms for sirolimus (A) and ascomycin (B) (internal standard). The ratio (R) of the integrals (A/B) is used to quantitate drug concentration for a given sample based on comparison to the linear standard curve for R values obtained for preparations of standards (C).

parameters for each analyte specified in the MRM. From a technical standpoint, the assay is then easy and fast to perform. Once a standard curve has been generated, results from a single sample can be obtained within ten minutes. Furthermore, LC-MS/MS assays perform with better precision than immunoassays, and the linear range of the assay can be adjusted to cover more than two orders of magnitude in drug concentrations.

Currently, calibrators/standards are available from at least two commercial sources (7), and control materials are available from at least three sources. Labs can perform proficiency testing via regular CAP surveys and also through a monthly international proficiency testing survey program. Laboratories should note that the interlaboratory variability in both surveys can be quite large in comparison to intralaboratory, interassay CVs for LC-MS/MS assays as reported in the literature. This variability is likely due in part to the combination in the surveys of results based on different commercial or in-house standards.

Startup Costs: The Business Considerations

In addition to technical considerations, a number of other considerations also impact a laboratory's decision of whether to implement LC-MS/MS for immunosuppressant drug monitoring. Initially, our institution was driven to consider acquisition of LC-MS/MS capability by the need to provide timely results for sirolimus monitoring before a sirolimus immunoassay had become commercially available. Economics played a large factor in this decision. Despite the rather large capital investment required, our business plan for acquisition of LC-MS/MS was extremely favorable due to the anticipated test volume and the high costs of comparable immunoassays.

We also anticipated in our business plan the cost of testing for new drugs. For example, in anticipation of U.S. approval of the immunosuppressant everolimus, we recently added the drug to our menu by simply modifying the MRM table. In comparison to the cost-per-test of an immunoassay, the inclusion of everolimus in the LC-MS/MS procedure incurs only a small additional cost to the operation of the assay.

Service issues are a primary concern for routine use of LC-MS/MS in the clinical laboratory. Although down-time due to fairly routine LC and autosampler issues can be

handled in-house, any failure of the MS/MS generally requires outside technical service. It is fair to say that, to the extent that routine use of LC-MS/MS in clinical laboratories is a relatively recent and growing development, the clinical laboratory and its service needs are a correspondingly novel arena for the technology vendors themselves. Service contracts for LC-MS/MS akin to those for STAT laboratory technology platforms are not generally available. Consequently, our business plan called for acquisition of two LC-MS/MS units. Our operating experience has been such that we would strongly recommend this option to those considering acquisition of LC-MS/MS.

An additional major consideration before implementing this type of laboratory service is the need for advanced technical expertise to maintain and operate MS/MS on a routine basis. There are no turn-key LC-MS/MS assays, and startup of LC-MS/MS involves a steep learning curve, even for the software alone. Although vendor-based operator training will be a part of the purchase price of a new LC-MS/MS system, such training likely will not be targeted to any particular application. Therefore, we recommend that labs consult with or arrange for training with an institution already performing immunosuppressants measurements using the exact same technology and software beforehand.


Other Laboratory Applications

Having LC-MS/MS in the clinical laboratory also presents the opportunity to utilize the technology for measurement of a wide array of analytes in addition to immunosuppressant drugs. The published literature contains many reports on therapeutic drug monitoring and drugs-of-abuse screening by LC-MS/MS (8). The 2005 AACC Annual Meeting included numerous presentations on tandem mass spectrometry, such as a full-day workshop chaired by Ravinder Singh, PhD of Mayo Clinic, who discussed measurement of drugs, hormones, and the large array of analytes associated with screening and diagnosis of metabolic disorders. Examples from metabolic testing include amino acid analysis by neutral loss scanning (a Q1 scan of ions exhibiting the neutral loss in Q3 of a common derivatized fragment) and acylcarnitine analysis (used to detect fatty acid oxidation disorders) by precursor scanning (a Q1 scan of all precursors that produce carnitine in Q3) (9).

The horizon for expanded use of LC-MS/MS in the clinical laboratory is indeed broad. For most clinical laboratories, the factors limiting use of the LC-MS/MS for additional assays are mainly available instrument throughput time and the time available for development and validation of methods.

MS/MS in the Clinical Lab: A Testament to Science

Measurement of immunosuppressants by LC-MS/MS represents the fundamental work of the clinical chemistry laboratory: turning blood into numbers. From the perspective of the end user, how those numbers are generated is rarely of any concern. Within the laboratory, however, we can marvel at the details and the technology.

The development of the MS/MS is a grand testament to basic science—to the genius involved in the conception and development of the quadrupole mass filter, the triple quadrupole instrument, the electrospray source and their applications in analytical chemistry; and to the advances in electronics, materials, engineering, and computing that are combined to produce a modern mass spectrometer. These advances have made the routine measurement of immunosuppressants by LC-MS/MS possible in the clinical laboratory. Given the power of the technology, numerous other exciting and beneficial applications of MS/MS are likely to become routine in the relatively near future. 

REFERENCES

1. Shaw LM, Holt DW, Keown P, Venkataramanan R, Yatscoff RW. Current opinions on therapeutic drug monitoring of immunosuppressive drugs. *Clin Ther.* 1999; 21: 1632–1652.
2. Taylor PJ. Therapeutic drug monitoring of immunosuppressant drugs by high-performance liquid chromatography-mass spectrometry. *Ther Drug Monit.* 2004; 26: 215–219.
3. Volosov A, Napoli KL, Soldin SJ. Simultaneous simple and fast quantification of three major immunosuppressants by liquid chromatography-tandem mass spectrometry. *Clin Biochem.* 2001; 34: 285–290.
4. Annesley TM. Ion suppression in mass spectrometry. *Clin Chem.* 2003; 49: 1041–1044.
5. Annesley TM, Clayton LT. Quantification of mycophenolic acid and glucuronide me-

tabolite in human serum by HPLC-tandem mass spectrometry. *Clin Chem.* 2005; 51: 872–877.

6. Vollenbroeker B, Koch JH, Fobker M, Suwelack B, Hohage H, Muller U. Determination of cyclosporine and its metabolites in blood via HPLC-MS and correlation to clinically important parameters. *Transplant Proc.* 2005; 37: 1741–1744.

7. Annesley TM. Application of commercial calibrators for the analysis of immunosuppressant drugs in whole blood. *Clin Chem.* 2005; 51: 457–460.

8. Maurer HH. Multi-analyte procedures for screening for and quantification of drugs in blood, plasma, or serum by liquid chromatography-single stage or tandem mass spectrometry (LC-MS or LC-MS/MS) relevant to clinical and forensic toxicology. *Clin Biochem.* 2005; 38: 310–318.

9. Chace DH, Kalas TA, Naylor EW. Use of tandem mass spectrometry for multianalyte screening of dried blood specimens from newborns. *Clin Chem.* 2003; 49: 1797–1817.

ADDITIONAL RESOURCES

A history of mass spectrometry is provided by the Scripps Center for Mass Spectrometry (<http://masspec.scripps.edu/information/history/>). An excellent on-line tutorial for mass spectrometry is provided by the Mass Spectrometry Research Center of Vanderbilt University (<http://www.mc.vanderbilt.edu/msrc/tutorials/index.php>). (Sites accessed December 2005).



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