You Should Have the Same Area Counts

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Mass spectrometry is a sensitive technique used to detect, identify, and quantify molecules based on their *m/z*. High-performance liquid chromatography (HPLC)[4] is a common separation technique used before analysis of biological samples by LC-MS/MS. LC-MS/MS is preferred over immunoassay for therapeutic drug monitoring of immunosuppressive drugs due to its specificity and sensitivity in detecting analytes in liquid and nonvolatile samples. However, time-consuming sample preparation and interferences resulting from ion suppression are among the disadvantages of LC-MS/MS because they can compromise or invalidate results. Also, although the instruments used are more robust, computer-interfaced, user-friendly, and easier to maintain compared with decades ago (*1*), their technical complexity leads to difficulties in troubleshooting and can be intimidating to laboratorians. Creating exhaustive system checklists and detailed maintenance are required to troubleshoot the nature of problems and solve them. With this reflection, we would like to share an unusual experience that confused us for some time.

We use LC–electrospray ionization–MS/MS for monitoring the immunosuppressive drugs cyclosporine, tacrolimus, sirolimus, and everolimus. Unique deuterated internal standards (ISs) for each drug are used to overcome issues caused by sample preparation and to detect ion suppression by observing the peak area counts for the ISs.

During post-run data inspection, we realized that the IS peak area counts were scattered; the cyclosporin calibrator IS areas were increased while tacrolimus calibrator IS areas were decreased, and the CV percentage (CV%) had exceeded the desirable value of 10% for all 4 drugs (*2*) (Table 1). If only 1 sample had a dissimilar area count compared to the rest of the samples, it would mean something related to the sample itself would be causing ion suppression. However, our data presented a generally increased scatter of area counts, so we suspected a general assay or instrument-dependent issue. Thus, we ran a check of all system components to diagnose the root cause of this previously unseen problem.

First of all, it was necessary to differentiate whether the problem was related to sample preparation, chromatography, or mass spectrometry with system suitability testing (SST) and direct MS/MS infusion. SST was performed using pure standards of the above-mentioned drugs (Eureka Lab Division); the sample preparation phase was bypassed to find out whether the problem had originated in the preparation phase or in one of the instrument components (*3*).

After serial injections of pure standards, the observed peak area CVs were higher compared to

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[4] **Nonstandard abbreviations:** HPLC, high-performance liquid chromatography; IS, internal standard; CV%, CV percentage; SST, system suitability testing.

Table 1. Deuterated IS area counts for each immunosuppressive drug before and after relocation of LC-MS/MS.

Before relocation					After relocation				
Sample name	Cyclosporine IS area	Tacrolimus IS area	Sirolimus IS area	Everolimus IS area	Sample name	Cyclosporine IS area	Tacrolimus IS area	Sirolimus IS area	Everolimus IS area
Cal 1[a]	154669	230532	102431	95278	Cal 1	194842	322289	160381	132800
Cal 2	161627	245842	108349	99116	Cal 2	127134	301461	102256	187903
Cal 3	177174	248361	103391	99560	Cal 3	289923	73967	190457	134756
Lvl 1	154976	247950	108145	102481	Lvl 1	356540	67923	87145	240675
Lvl 2	146001	234842	104009	97235	Lvl 2	176343	185723	70296	289120
Patient 1	173168	231601	99797	97499	Patient 1	149452	201982	259222	64923
Patient 2	145888	207354	93763	86325	Patient 2	206591	358461	159250	77881
Patient 3[b]	139183	210582	96386	93823	Patient 3	195824	100386	94729	138934
Patient 49	169329	201024	90571	86513	Patient 76	178945	233700	90571	86513
Patient 50	128030	208270	91056	95721	Patient 77	159622	281230	91056	95721
Patient 51	138431	204620	90409	85361	Patient 78	182581	86921	120600	85361
Mean	145657	215396	96178	92915	Mean	221945	231862	133211	125352
SD	13728	10728	4096	3852	SD	65917	106921	57188	72144
CV%	9.4	5.0	4.3	4.1	CV%	29.7	46.1	42.9	57.6

[a] Cal, calibrator; Lvl, level of controls.
[b] The 3rd to 49th and 3rd to 76th patients' data are not shown in the before and after relocation columns, respectively, but 51 and 78 patients' data are included in the calculation of SD and CV% of both groups.

previous SST values, which indicated that the problem was not related to sample preparation.

For evaluation of the MS/MS piece of the measurement alone, reserpine (Sigma-Aldrich) was injected directly into to the module, bypassing the sample preparation and chromatography phases. The scatter persisted, indicating that we were facing a problem originating in the MS/MS component. If the problem had originated from the autosampler, we would have expected the variations to be systematic and in the same direction. During a step-by-step review, we cleaned the interface region, where residual matrix deposits can accumulate and cause loss of response; we checked all inputs and other details such as MS/MS gas pressures (N_2/Ar); and we checked the oil level and pressures of the vacuum pump, environmental contaminants, computer maintenance, and mass calibration/tuning. Despite all these interventions, the problem persisted.

After these vigorous rechecking procedures, the vendor technical support group suspected an electrical supply problem and checked the grounding of the power source, which was 8 times higher than the maximum allowable voltage (<1 V). It became apparent that this problem had resulted 1 week ago during the relocation of the LC-MS/MS to another room within the laboratory where other HPLC systems were being used without any problem. When we investigated why we did not have this problem previously, we learned that there were 3 different power sources in the hospital. The new room's power source was different from the rest of the laboratory and was not properly grounded. After reverting to the old powerline, the IS peak area count CV% diminished to desired levels.

Electrical troubles are among the most difficult to detect in laboratories. Ochran and Konermann reported that grounding affects ionization of analytes in electrospray ionization mass spectrometry and that most LC-MS/MS users are unaware of the electrochemical consequences of grounding (*4*). Grounding affects ionization, leading to variations in the peak areas for all 4 drugs we were measuring and their ISs; these errors were not systematic and in the same direction. Consequently, calculated concentrations of both analytes and QC samples were affected because the ISs were inadequate for properly correcting the variations in ionization. We concluded that improper grounding was the cause of this problem, leading to fluctuations in signal intensities and causing high CV% values. Previously, our validation plan for setting up and relocating devices mostly included instrument-based maintenance programs, since the electrical sources were monitored by hospital technical services.

In conclusion, after major instrument maintenance or replacement of parts, power outages, and changes in environmental conditions, the performance of LC-MS/MS should be checked. Also, an uninterruptible power source should be used to prevent interruptions during loss of power whenever feasible (*5*). While setting up a mass spectrometry laboratory, site preparation should involve meeting the manufacturer specifications regarding the requirements for space, electrical source, environmental contaminants, gas supplies, and temperature control. According to the manufacturer, the line powering LC-MS/MS should be adequately grounded to avoid charging and the risk of exposing the operator to high voltages. Also, it is recommended that clinical laboratories should have a validation plan encompassing all technical issues.

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