Troubleshooting Cases from a Large Reference Laboratory

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Troubleshooting

• Important aspect of patient safety and risk management

• Instrument, people issues, materials, and method
Polling question

• Which is more difficult in your lab

• Select “yes” for day-to-day routine operations

• Select “no” for troubleshooting problems
Case 1: Why am I failing proficiency testing (PT)? (metanephrines)
Metanephrines PT program

• Peer-group based (i.e. consensus-based), not accuracy-based

• PT samples
  – lyophilized urine
  – reconstituted
  – taken through the same sample-processing steps as patient samples
The problem

• Lab M failed three consecutive PT rounds
• Results from lab M all came out high
• Most other labs generally passed PT and agreed well amongst themselves
Polling Question

• Which lab or labs were giving more correct results?

• Vote “yes” for peer group

• Vote “no” for lab M
Investigation

• CAP asked 3 highly reputable labs to investigate
  – Lab M
    • Clinical testing lab that was failing PT
  – Lab N
    • Lab at a government agency
  – Lab A
    • Clinical testing lab that was passing PT
Possible explanations

– Matrix bias
– Non-commutable PT samples
  • Proficiency testing samples used non-native samples (lyophylized urine)
– Interfering compounds
– Wrong compound detected
  • Wrong mass setting in MS
  • Wrong LC peak integrated
– Bad calibrators
– Labeling error in lab
– Failure to follow SOP
Polling Question

- What do you think is the problem?
- Vote “yes” for bad calibrators
- Vote “no” for failure to follow SOP
Calibrators used by labs in PT program

• Before early 2004, only 1 commercially available source of urine calibrators
  – From Company B
  – Most labs used these calibrators
  – Lyophylized urine
  – Reconstituted before use

• Lab M made weighed-out calibrators from pure metanephrines
  – Added to 20% methanol
Method comparison for investigation

- Each laboratory used a different method of analysis
  - Lab M:
    - LS-MS/MS
    - Weighed out calibrators
  - Lab N:
    - HPLC-EC
    - Weighed out calibrators
  - Lab A:
    - GC-MS
    - Commercial calibrators from company B
Calibrator properties

- **Lab M**
  - Weighed out from pure materials
  - Source: Sigma
  - Matrix: 20% methanol

- **Lab N**
  - Weighed out from pure materials
  - Source: Sigma
  - Matrix: 0.2% acetic acid

- **Lab A**
  - Commercial calibrators
  - Matrix: reconstituted lyophylized urine

- Matrix effects?
Calibrator comparison strategy

• Lab M
  – Ran commercial calibrators as “unknowns” using their method (using weighed out calibrators)

• Lab N
  – Ran commercial calibrators as “unknowns” using their method (using weighed out calibrators)

• Lab A (two experiment types)
  – Samples prepared in house by weighing pure materials and run as “unknowns” (using commercial calibrators)
  – Ran calibrators from Lab M as “unknowns” (using commercial calibrators)
Polling Question (again)

• What was the problem?

• Vote “yes” for bad calibrators

• Vote “no” for failure to follow SOP
Conclusions from investigation

• Peer group was wrong
  – Faulty commercial calibrators (or possibly adjusting their calibrations to be consistent with commercial calibrators)

• Lab M was right
  – Accurate weighed out calibrators

• Commercial calibrators were ~24-33% lower than gravimetrically based concentrations
  – Consistent finding from labs A, M, and N
Possible weaknesses of investigation

- Matrix effects of calibrators not fully evaluated
Sources of samples of “known” concentration

• Certified reference materials

• Gravimetrically prepared samples from pure materials

• Previously run PT samples
Recent PT results

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- Harmonized results (commercial calibrators improved)
Some take home lessons

• When obvious explanations don’t work cast a wider net!
• Don’t assume passing PT means you are “right”!
• Don’t assume failing PT means you are “wrong”!
• Don’t assume all standard materials are equally valid!
• Outside help is sometimes necessary!
• Don’t give up!
Precisely wrong? Urinary fractionated metanephrines and peer-based laboratory proficiency testing.

Article in Clinical Chemistry 51(2):472-473; response 473-4 · February 2005
Case 2: Where is all the estrone coming from?
Analytes, method, and background info

- Estradiol and estrone by LC-MS/MS

- High sensitivity Estrogen assay transitioned from R&D to production lab

- 1 year later, problems started
The problem

• Runs suddenly started to fail for estrone
  – Negative controls failed (2-3 pg/ml)
  – “Good” chromatographic peak at correct estrone retention time

• Turnaround time and quality suffered
Chromatograms unremarkable...

... except that negative controls showed elevated “estrone”
Trouble shooting philosophy

• First, look for the obvious
  – People issues
  – Empty mobile phase bottles
  – Source gas not turned on
    Tubing not connect or leaks
  – Etc.
• Then start at beginning of flow path (even before sample hits the instrument)
• Follow the flow path (problems tend to be more expensive the further down the path the problem is.)
Polling question

• Given that the method started suddenly, which seems more likely?

• Select “yes” if it is a “people issue”
  – (insufficient training, failure to follow SOP, etc.)

• Select “no” if it is a problem with consumables
Preliminary steps

• Following SOP?
• Training?
• Good analytical technique?
• Pipettes and vials?
  – calibration
  – contamination
  – not re-using pipette tips, etc.
• Transfer of aqueous fraction (frozen) to 96 well plates? (A method-specific issue)

Image source: http://www.cliparthut.com/science-fair-clip-art-clipart-P6DaRN.html
Investigations

• First steps: ruled out “people issues”
  – Staff adequately trained
  – Staff following SOP
  – Etc.
Investigations

• New mobile phases made
• Needle and needle wash contamination?
  • Wash cycle
  • Wash solution contamination
  • Making new solutions
• Injection port carryover?
  • Disassembled injection valve
  • Checked for tubing contamination
Investigations (continued)

- Changed guard column & run test injections
- Back flushed column
- Looked for leaks in HPLC
- Mass spectrometer
  - Capillary in nebulizer installed correctly
  - Curtain plate cleaning
  - Etc.
Head scratching time

- Checked with R&D staff and medical directors
  - No new insight
- Testing on delay
- Time to think “outside of the box”
Astute tech looked at supplies e.g. plates and mats
Archimedes moment

- Checking supplies
  - Extracted plate using MTBE – no luck
  - Extracted plate mat with MTBE and ran on mass spec

Eureka!!!!
Outcome

• Vendor of mats had switched to a new plastic material
  – Contaminated

• Switched vendors – problem resolved

• Ideally, would have researched what the contaminant was, etc., but production schedule took precedence
Lessons learned

• Trouble shooting can be long & difficult
• Work your way from front to back
  – People issues
  – Front of process/instrument to back end
  – Most obvious causes first
  – Then let your imagination fly
• Technologists can often supply crucial insights and flashes of inspiration
Lessons learned - continued

• “Harmless” and subtle changes of consumables can cause major problems
  – Uncommon but not rare
  – Lot to lot variation can be serious
  – Lot Validation may be required for critical components to maintain quality
  – Standardization of primary lab supplies helps identify problems early
  – Lot sequestration of supplies is helpful if you have leverage with a vendor
Case 3: Why are we getting false positives?

Case courtesy of Fred Strathmann
Analytes, method, and problem

• Estradiol and estrone

• LC-MS/MS

• Sporadic false positives for estradiol
  – Client reports that results are not consistent with the rest of the clinical picture
  – Re-runs did not confirm results of first analysis on some fraction of the samples
Trouble shooting

• Skipping over the details, ultimately investigation focused on cross contamination between samples in 96 well plates
Investigation focus

- Samples adjacent to elevated samples in 96 well plate were most likely to show this problem

Elevated “hot spot”

Samples at risk
What’s going on?
Transfer between wells?
Are you kidding me?
From ASMS 22015 poster: Evaluating the Potential for Cross Contamination when performing 96-well Sample Preparation prior to LC-MS/MS Analysis by Helen Lodder et al.
Mitigation strategy #1 (procedural)
Mitigation strategy #1

“Tweener” concentration

Slide courtesy of Fred Strathmann
Mitigation strategy #1
Mitigation strategy #1

Re-extract tweener sample
Mitigation strategy #2 (engineering)

Plate adapter on top of 96 well plate

From ASMS 22015 poster: Evaluating the Potential for Cross Contamination when performing 96-well Sample Preparation prior to LC-MS/MS Analysis by Helen Lodder et al.
Mitigation strategy #2 (engineering)

Plate adapter redirect flow of drying gas

Figure courtesy of Fred Strathmann
Polling question

• Which type of problem mitigation do you generally prefer?

• Select “yes” for procedural

• Select “no” for engineering
Lessons learned (Case 3)

- After all obvious causes have been eliminated, the unlikely explanation becomes a candidate
- QC rules don’t always catch the problem
- Listen to client complaints, then investigate
- Multiple mitigation strategies are often possible
  - After the fact vs. before the fact
  - Procedural vs. engineering
  - Etc.
Grand strategy concepts
Use QC effectively

• QC rules should fit the assay

• Repeat vs. investigate?

• Type of QC failure often correlates with type of underlying problem
Using Westgard rules

• Customizing QC rules to fit the assay

• Best practices
  – https://www.westgard.com/lesson74.htm

• Ten ways to go wrong

• On eliminating the 2s trigger rule
Polling question

- Which is the more frequent source of problems in your LC-MS/MS lab?

- Select “yes” for the LC system

- Select “no” for the mass spectrometer
Troubleshooting prioritization

• 60% of problems are related to the LC and 40% MSMS
• Defining Limits of intervention before its too late!!!
  – End users
    • Simple pump or LC problems
    • Don’t go past Q zero
    • NO Method modifications
  – Senior Technologist or R&D Staff (Is it Instrument or Method?)
    • Tuning
    • Method modifications (document and validate)
    • Chemistry (columns and mobile phase)
    • Mechanical systems and plumbing
    • No power supplies. (25 to 75 kV)
    • No electronics
  – Service Engineers (in-house or external)
    • Electronics
    • Vacuum Systems (know what you are doing or it’s a $15,000 mistake)
    • Power supplies (fully trained staff only)
    • Quads (pulling the rail)
Hot tip!
Use ratio of two MS/MS transitions to detect interferences
Keep and use records

• Action report by Admiral Nagumo,

The Japanese Story Of The Battle Of Midway

OPNAV P38/1002

• After major battles many armies and navies prepare a report, including “lessons learned”

• Let us do the same with our troubleshooting.

Translated by Fred Woodrough, Jr.
Learn from the past!

• KEEP A TROUBLESHOOTING LOG BOOK.
• Write and keep “Lessons Learned” documents.
• Periodically review the logbook and lessons learned document to refresh your memory and to refine your troubleshooting flow charts.
• Develop troubleshooting SOPs and flowcharts.
• When troubleshooting, refer back to the above documents.
Polling question

• Have you started developing troubleshooting SOPs in your lab?
  • Select “yes” for yes
  • Select “no” for no
Useful resources:

• Mass spectrometry troubleshooting guide: [ww2.chemistry.gatech.edu/~bostwick/stms/spec.html](ww2.chemistry.gatech.edu/~bostwick/stms/spec.html)
• Mass spectrometry troubleshooting guide for beginners: [www.mass-spectrometry-news.com/tutorials/mass-spectrometry-troubleshooting-for-beginners/](www.mass-spectrometry-news.com/tutorials/mass-spectrometry-troubleshooting-for-beginners/)
Useful resources:

• LCGC Guide to LC Troubleshooting wallchart
An Individual Behind Every Sample
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