Patient Centric Approach for Run Acceptance Criteria in Large Targeted Metabolomics Panels

Sally Abou-Zeki, Pamela Dartnall, Melissa J. Bennett, Ahmed Najar, George Gillson

Rocky Mountain Analytical Division of LifeLabs LP, Calgary, Alberta, Canada

Ahmed.Najar@lifelabs.com

Overview:

Purpose: Develop a fast and patient centered approach to approve batch run for a panel of 31 steroids in a clinical lab setting

Methods: Intra-batch acceptance was based on a pooled sample from the same patients run on that day

Inter-batch acceptance was based on four samples (pool and lyophilized)

For both intra- and inter-batch validation, principal component analysis (PCA) was used along with D-Ratio (a variance based metric)

Results: PCA and D-Ratio provided a fast and quantifiable way to pinpoint issue- compounds

This approach does not account for the positive or negative bias like Westgard rules do. We suggest a combination of Westgard and PCA for batch acceptance

Introduction:

• As the need for large diagnostic panels in clinical chemistry rises, the use of Westgard rules is causing the failure of batches that could otherwise pass.

• Lyophilized Certified Reference Material and spiked matrices do not account for patient matrix and rarely contain all endogenous compounds. The FDA guidelines for bioanalytical method development and validation[1] proposes a patient centric approach that should account for these limitations.

• Run acceptance has two elements in the clinical practice: (1) intra-batch to monitor preparation and instrument stability and (2) inter-batch to insure results from day to day are consistent.

• We propose a method to quickly assess run acceptance of a large panel of steroid metabolites.

Methods:

Constructed pools

Intraday pools: From the same patient samples run in the batch, equal volumes from every patient sample were pipetted and mixed in a polypropylene reservoir. From that pool, 4 samples were processed as individual samples. The 4 replicates account for user variation as the method requires several steps (Figure 1). Those 4 replicates will be referred to as QCM since their variance translates the reproducibility of the sample preparation. These 4 samples are injected multiple times throughout the batch (total 12 injections) in order to monitor instrumental variability. The replicate injections from the 4 QCM are referred to as QCR (repeats of the QCM). The intra-batch validation data is not shown here.

Inter-day pools: a total of 4 QC samples were used:

- Two are lyophilized (1) Bio-Rad Lyphocheck L-1 (Montreal, QC) and (2) SKML SRM (Nijmegen, Netherlands).
- Two were pooled from a pregnant female (for estimable amounts of estrogens) and a male below 30 years old at different ratios. We labeled them as CHI and CHI2 (CHI: Comprehensive Hormone Insights®).
- The present work reports data from 10 batches run over a month.

Sample Preparation

We use an original in-house sample processing method consisting of an enzymatic hydrolysis (β-glucuronidase/arylsulfatase from *Helix pomatia*) in order to convert the glucuronide and sulfate conjugates to their unconjugated steroid unit. The samples are cleaned using polymeric solid phase extraction followed by a derivatization using N-methyl-N-(trimethylsilyl)-trifluoroacetamide.

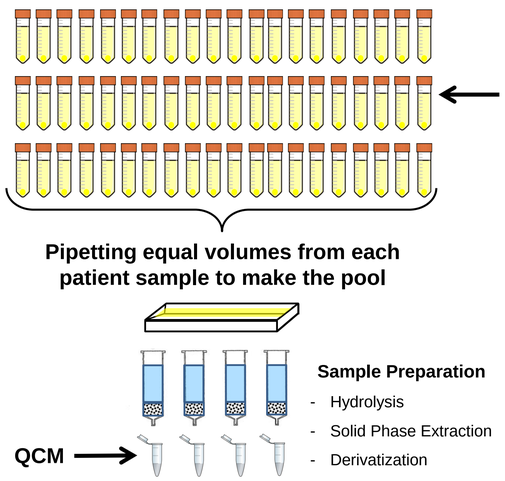

Figure 1: Scheme for patient centric pool construction. **QCM** are method replicates. **QCR** are replicate injections.

Methods continued:

GC-MS/MS analysis

Urine steroid analysis using gas chromatography is the gold standard in clinical chemistry particularly for a large panel with different ionization requirements in a LC-MS/MS setting.

Instrumentation

- Agilent 7890B Gas Chromatogram
- Agilent 7000D Triple Quadrupole
- Column VF-200ms
- 7000D was used in dynamic MRM mode and unique transitions were used for mass separation of co-eluting compounds
- Representative compounds from androgens, corticoids, estrogens and β-pregnanediol were analyzed

Analytics

PCA was performed using the `vegan` package in R. D-ratio, a measure from untargeted metabolomics QC[2] provides an estimate of instrumental variance relative to biological variance.

$$D-ratio = \frac{\sigma_{technical}}{\sqrt{\sigma^2_{samples} + \sigma^2_{techniqual}}} \times 100$$

Where:

$$\sigma_{technical} = variance\ of\ pooled\ QC\ both\ QCM\ and\ QCR$$

$$\sigma_{samples} = variance\ of\ patient\ samples$$

If D-ratio ≤ 5% then the technical variation is negligible, if D-ratio = 100 then the biological variation is negligible (not likely and triggers an investigation of the issue).

Results:

Graphic assessment shows decent clustering of all QC samples (Figure 2). The ellipses represent the 95% confidence interval, which is wider for QCM and QCR since the samples vary from day to day. The perfect overlap of QCM and QCR shows instrument stability over a month worth of runs. The D-ratio from QCM and QCR is assessed on a daily basis and showed satisfactory results (data not shown).

Inter-day assessment of method stability using QC values shows good clustering and D-ratio values that meet the specification (<5%). Cases that did not meet the D-ratio specifications were flagged and investigated.

References:

[1] U.S. Department of Health and Human Services Food and Drug Administration (2018). Bioanalytical Method Validation Guidance for Industry (https://www.fda.gov/media/70858/download), [2] Broadhurst , D. et al. (2018). Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. Metabolomics 14:72.

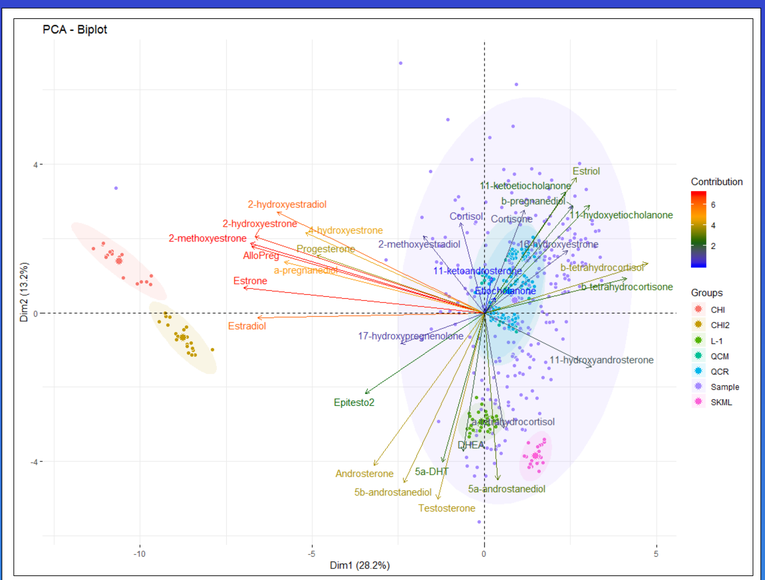

Figure 2: Graphical estimation of variance of intra and inter day QCs.
CHI and **CHI2** are pools made from different ratios of a pregnant female and a male with high androgen levels; **L-1** and **SKML** are lyophilized urine steroid reference material; **QCM** and **QCR** are pooled samples prepared on the same day; **Sample** are 200 patients (50 males and 150 females: 50 menopause, 100 cycling women in both parts of their cycle)

Compounds above the calibration range lack precision and show high variance (flagged in orange). Similarly to compounds below limit of quantitation (LOQ flagged in blue). This method successfully identified compounds that were not properly integrated due to matrix effects influencing the user integration (Table 1).

Table 1: Summary of D-ratio from the four interday QCs. below LOQ, above calibration range, user integration inconsistency.

Compound	D Ratio CHI	D Ratio CHI2	D Ratio L-1	D Ratio SKML
11-hydoxyetiocholanone	0.15	0.00	0.12	0.40
11-hydroxyandrosterone	0.99	0.33	3.06	
11-ketoandrosterone	0.36	0.01	0.12	0.47
11-ketoetiocholanone	0.85	0.01	0.31	0.34
16-hydroxyestrone			0.55	0.01
17-hydroxypregnenolone	5.83	3.83	3.43	4.69
2-hydroxyestradiol		2.45	0.13	
2-hydroxyestrone			0.02	0.28
2-methoxyestradiol	0.07	0.02	0.00	
2-methoxyestrone			0.09	
4-hydroxyestrone			1.38	
5α-androstanediol	0.25	0.03	0.41	2.87
5α-DHT	1.55	0.27	1.34	3.25
5β-androstanediol	0.88	0.18	0.41	2.32
α-pregnanediol		1.37		
α-tetrahydrocortisol	0.42	0.35	0.98	
Allo-Pregnanolone			0.01	
Androsterone	5.23	0.47	1.43	
β-pregnanediol			0.01	0.03
β-tetrahydrocortisol	0.26	0.05	0.37	2.97
β-tetrahydrocortisone	0.87	0.29	1.21	3.83
Cortisol	2.01	1.06	0.39	2.79
Cortisone	1.55	1.38	0.27	1.82
DHEA	0.02	0.50		0.31
Epitestosterone	5.40	0.66	0.47	4.85
Estradiol				0.23
Estriol				0.21
Estrone			1.14	0.38
Etiocholanone	1.40	0.23	0.77	3.07
Progesterone				
Testosterone	0.29	0.02	0.28	0.23

Conclusions:

This approach to run acceptance accounts for patient matrix in clinical samples and provides another layer of review beyond more conventional metrics (recovery, ion ratios, etc.)