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Challenges of Cross-Lab Validation for Sphingolipids Biomarker Assays with Multiple Isomers in Human Dried Blood Spots

Allena J. Ji[1], Nan Zhao [2], Yi Zhu[1], Mona Hdeib[1], Troy Voelker[2], and Scott Reuschel[2]

[1]Sponsor, Biomarkers and Clinical Bioanalyses-Boston, Sanofi, Framingham, MA, USA, [2]Covance, Salt Lake City, UT, USA

CONTACT INFORMATION: Allena.Ji@sanofi.com.

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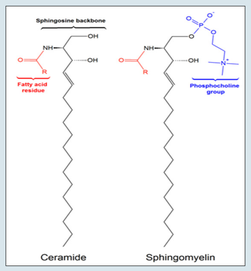
PURPOSE

Total ceramide (11 isomers, CER) and total sphingomyelin (17 isomers, SPM) (Figure 1) are under evaluation as biomarkers in human dried blood spots (DBS) for monitoring patients with Niemann-Pick disease, who have received enzyme replacement therapy. The two bioanalytical methods used for several clinical trials in the sponsor's lab were transferred to Covance. Both methods were cross-validated at Covance and met precision and accuracy acceptance criteria using purified porcine CER or SPM as reference standards. However, bridging normal reference ranges from the same 100 human individual blood samples showed significant difference between the two labs for total CER and total SPM. Systematic troubleshooting with innovative approaches was conducted, and results showed that the different isomer patterns between porcine and human analytes caused the problem. Modification of instrument collision energy to match isomer patterns of porcine CER and SPM in DBS standards between labs is necessary to obtain comparable human reference values between labs.

Figure 1. Structure of ceramide and sphingomyelin



METHOD(S)

Dried blood spot (DBS) samples were punched into a 96-well plate and soaked in the solvent with IS. The supernatant was transferred to a clean plate for LC–MS/MS analysis. Sample analyses were performed on Sciex API 4000 with Aquity UPLC, by using isocratic mobile phase and a reverse phase HPLC column. MRM mode was used for CER (11 isomers and its IS for 12 transitions), and SPM (17 isomers and its IS for 18 transitions), respectively. All method procedures from the sponsor were followed. Stock solutions for CER and SPM were shared by the two labs. Mass spectrometer's parameters, including collision energy and decluttering potential, were adjusted through all isomers to match the isoform patterns of standards from the sponsor's lab. The chromatogram of total ceramide and total SPM in human dried blood spot containing endogenous are shown in Figure 4 and 5, respectively..

RESULT(S)

Figure 2. Chromatogram of a 4.00 µg/mL calibration standard (LLOQ (containing endogenous ceramide)): ceramide in human dried blood spot.

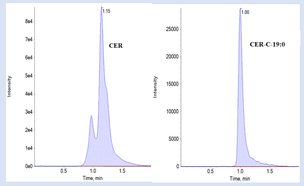


Figure 3. Chromatogram of a 500 µg/mL calibration standard (LLOQ (containing endogenous sphingomyelin)): sphingomyelin in human dried blood spot.

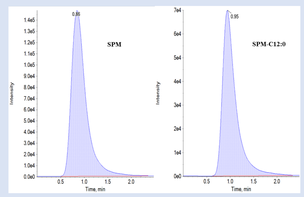


Figure 4. Different reference values from the same 100 donors for CER in human dried blood spots **before** CER method parameter modification using identical instrument parameters in both labs.

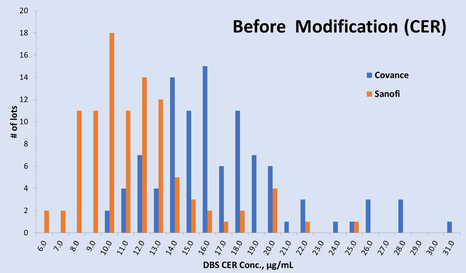


Figure 5. Different reference values from the same 100 donors for SPM in human dried blood spots **before** SPM method parameter modification using identical instrument parameters in both labs.

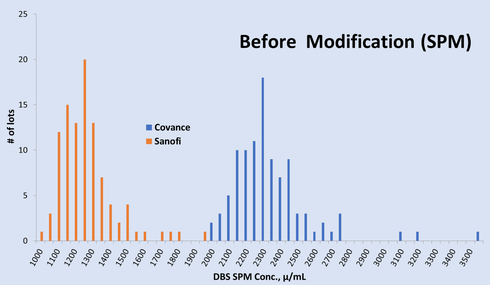


Figure 6. Comparison of % isomer profile pattern before and after adjusting CE and DP : SPM in human dried blood spot.

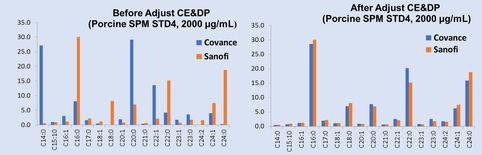


Figure 7. Comparison of % isomer profile patterns before and after adjusting CE and DP: CER in human dried blood spot.

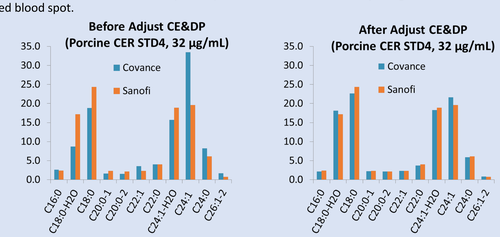


Figure 8. Overlapped ceramide reference values from the same 93 donor DBS samples **after** CER instrument parameters' modification to match sponsor's isoform pattern in porcine CER standards.

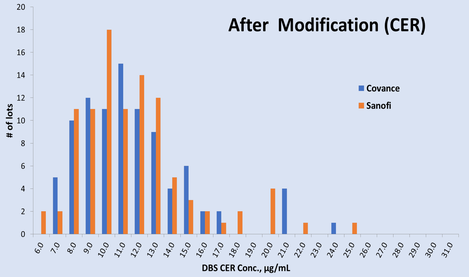
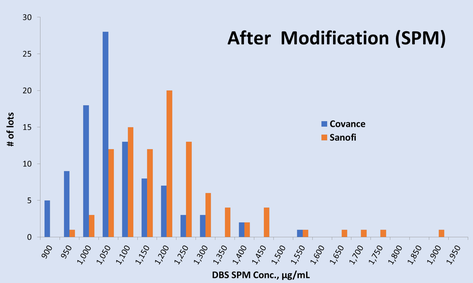


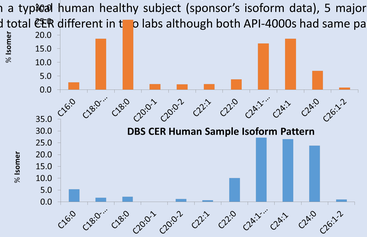
Figure 9. Overlapped sphingomyelin reference values from the same 100 donor DBS samples, **after** SPM Instrument parameters' modification to match sponsor's isoform patterns in porcine SPM standards.



Precision and accuracy of both assays met the validation acceptance criteria when standards and QCs shared the same porcine reference standard for each assay. After completion of the majority of validation parameters, reference values from 100 healthy donors were evaluated and showed significant differences between the two labs when the same stock solutions and same instrument parameters were shared. For DBS CER, the sponsor's lab result was 12.1 ± 3.43 µg/mL (Mean ± SD), while Covance's original result was 17.3 ± 4.23 µg/mL (Mean ± SD) (Figure 4). For DBS SPM, the sponsor's lab result was 1240 ± 161 µg/mL (Mean ± SD), while Covance's original result was 2314 ± 237µg/mL (Mean ± SD) (Figure 5).

Our investigation showed that the different values between labs were caused by the differences in isoform patterns between natural purified porcine CER or SPM as reference standard and endogenous human CER and SPM as analytes (Figure 6 and 7). For SPM, adjustment of collision energy was sufficient to match isoform patterns in DBS standards between labs. The final SPM result became 1096 ± 109 µg/mL (Mean ± SD), which is similar to the sponsor's result (Figure 9). For CER, because the reference standard's isoform pattern was significantly different from endogenous human CER (Figure 10), adjustment of collision energy and decluttering potential to match sponsor standard's isoform pattern was not enough. The same lot of pre-spotted human DBS cards with low endogenous CER was also required for standard curve preparation. The final CER result was 12.1 ± 3.40 µg/mL (Mean ± SD), which matched results from the sponsor's lab (Figure 8).

Figure 10. Comparison of CER isomer profile patterns between CER reference standard spiked on a pre-spotted human DBS with a typical human healthy subject (sponsor's isoform data), 5 major isoforms are different in abundance, which caused total CER different in two labs although both API-4000s had same parameters.



CONCLUSION(S)

The results of total sphingolipids in human blood can be significantly different between two labs due to isoform pattern mismatch between animals (for standards) and humans (unknown). Adjusting instrument parameters could lead to isoform pattern similarity and comparable reference ranges between labs.

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