CORESTA Biomarkers Sub-Group

Technical Report

Inter-Laboratory Comparison Test Measuring Total NNAL in Human Urine

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1. Introduction

In an ongoing effort to fulfill the second objective of the Biomarkers Sub-Group, an inter-laboratory comparison was organized to assess the consistency of laboratory quantitation when measuring total urinary concentrations of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). NNAL is the stable carbonyl-reduced butanol metabolite of 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK has been identified as carcinogenic to humans by the International Agency for Research on Cancer [IARC Monograph Vol. 89] and the U.S. Food and Drug Administration [Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke: Established List].

The measurement of total NNAL excretion has been established as a suitable biomarker to assess NNK exposure [1]. NNAL is excreted as the aglycone, an N-oxide and as both an N- and O-glucuronide. Most published assays focus on the measurement of "total" NNAL by the detection of the aglycone after hydrolysis of the N- and O-glucuronides [2]. All assays from participating laboratories in this inter-laboratory comparison followed this approach.

2. Field of Application

The laboratories participating in this comparison are all active in the analysis of clinical samples to support tobacco or tobacco-related product research.

3. Abbreviations and Terminology References

- **% C.V**.: Coefficient of variance
- **NNK**: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
- **NNAL**: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol
- **NNAL-*O*-Gluc**: 4-(Methylnitrosamino)-1-(3pyridyl)-1-butanol O-β-D-glucuronide
- **NNAL-*N*-Gluc**: 4-(Methylnitrosamino)-1-(3pyridyl)-1-butanol N-β-D-glucuronide
- **NIST**: National Institute of Standards and Technology
- **LC-MS/MS**: Liquid Chromatography coupled with tandem mass spectrometry detection
- **QC**: Quality control sample
- **% R.E.**: Relative error

4. Purpose

The purpose of this document is to summarize the inter-laboratory comparison study performed by the Biomarkers Sub-Group to determine the comparability of results reported from participating laboratories when analyzing total NNAL in human urine clinical samples.

5. Initial Participating Laboratories

The inter-laboratory comparison was initiated with the participating laboratories shown in Table 1.

Table 1. Initial participating laboratories

Laboratory	Contact	Address
Celerion – Lincoln[1]	Kirk Newland	624 Peach Street, Lincoln, NE 68502, U.S.A.
ABF GmbH[2]	Max Scherer	Goethestrasse 20, 80336 Munich, Germany
SEITA	Valerie Troude	SAL-LA Laboratory, 48 rue Danton, 45404 Fleury-les-Aubrais, France
University of Minnesota	Steve Carmella	233 6th Street SE, Minneapolis, MN 55455, U.S.A.
China National Tobacco Quality	Liu Tong	Supervision and Test Center, 2 Fengyang Street, Zhengzhou, Henan 450001, P.R. China
Zhengzhou Tobacco Research Institute of CNTC	Wang Sheng	No. 2 Fengyang Street, High-tech Zone, Zhengzhou, P.R. China
Shanghai Tobacco Group	Gang Li	717 Changyang Street, Shanghai 200082, P.R. China
KT&G Research Institute	Mi Jang	30, Gajeong-ro, Yuseong-gu, Daejong 34128, South Korea
Covance Laboratories	Johannes Stanta	Otley Road, Harrogate, North Yorkshire, HG3 1PY, U.K.

Prior to initiation of the inter-laboratory comparison study, an evaluation of the assay approaches and linear ranges was conducted to ensure comparability of the bioanalytical methods. It was confirmed that each laboratory performed a hydrolysis step to determine the total elimination of NNAL and the N and O-glucuronides by detection of the aglycone only. The hydrolysis was confirmed by each laboratory to be performed enzymatically with an optimized addition of β-glucuronidase. Each laboratory confirmed that detection would be performed by LC-MS/MS. An overlapping linear range of 5.00–1000 pg/mL was shared by all laboratories with the exception of one (35.0–1000 pg/mL).

6. Inter-Laboratory Comparison Design

The inter-laboratory comparison test to be completed by each laboratory required a single analytical run with a total of 60 test samples. The test samples were prepared at Celerion-Lincoln and shipped to each laboratory in duplicate to account for possible analytical failure. A description of the test samples is provided in Table 2.

[1] Organizing laboratory
[2] Updated address: Semmelweisstrasse 5, Planegg, Germany 82152

Table 2. Test samples

Sample Type	NNAL Form Present	Number of Aliquots Required for Test
Low concentration QC (15.0 pg/mL)	NNAL Only	6
Mid concentration QC (100 pg/mL)	NNAL Only	6
High concentration QC (750 pg/mL)	NNAL Only	6
Imbedded standards (9 concentrations)	NNAL Only	1 x 9 concentrations
Control blank samples	None	3
Different lots of smokers' urine	NNAL NNAL-O-Gluc NNAL-N-Gluc	3 x 9 lots
NIST urine standard	NNAL NNAL-O-Gluc NNAL-N-Gluc	3 x 1 lot

Each laboratory was tasked with the analysis of the above list of samples in an analytical run also containing calibration standards prepared at their own laboratory with their standard source of reference material. The organizing laboratory utilized NNAL purchased from Synthèse Aptochem Inc. (Lot No. AC0105005, Purity 99.9 %). Synthèse Aptochem provided the reference material in a qualified solution certified at 946 µg/mL. The reference materials utilized at the participating laboratories were not identified for this inter-laboratory comparison.

Control urine was collected from smoking and non-smoking consented volunteers at Celerion-Lincoln. The control blank urine was used to prepare quality control and test samples. The individual lots of smoker urine known to contain the aglycone and both glucuronides were used to create the 9 multiple lot test samples. The urine samples were stored at −20 °C. The NIST Urine Standard was purchased from the National Institute of Standards and Technology (SRM 3671, Certificate date 03-Dec-2015, Expiry 01-Jan-2020). The NIST Urine standard was stored at −80 °C both before and after sub-aliquotting as test samples. All samples were shipped to the participating laboratories frozen on dry ice.

The set of imbedded standards were prepared at 9 concentrations in control matrix (5.00, 10.0, 20.0, 50.0, 100, 200, 400, 800, and 1000 pg/mL). The imbedded standards were stored at −20 °C prior to shipment.

All samples were labeled with a numerical code. The laboratories performing the analysis were blinded to the identity of each sample. Each laboratory received results from the evaluation unblinded for their own data.

The reported results from each laboratory included the measured total NNAL concentration (pg/mL) as well as the analyte and internal standard area counts. The reported area counts allowed the regression of the measured test sample results against the set of imbedded calibration standards provided to each laboratory from the organizing laboratory.

7. Final Participating Laboratories

The inter-laboratory data comparison was completed with only four of the nine participating laboratories providing final data for evaluation. The participating laboratories were Celerion-Lincoln, ABF GmbH, SEITA and the University of Minnesota. The other laboratories were unable to provide results because they were either unable to receive biological samples shipped internationally, the qualified assay was not operational at the agreed time of testing, or the laboratory failed to communicate any results of analysis.

8. NNAL Result Comparison of Provided Quality Control Samples

Quality control samples prepared in control blank urine and supplemented with the aglycone NNAL only at concentrations 15.0, 100 and 750 pg/mL were assayed by each of the participating laboratories. This evaluation removes the hydrolysis step from consideration as a possible source of bias as no glucuronide metabolites were included in the testing. The laboratories were instructed to analyze each quality control sample with 6 replicates to allow evaluation of precision across the linear range of the assays.

The results provided by the participating laboratories with quantitation achieved with self-prepared standard calibrators demonstrated significant bias (greater than 15 %) at lower concentrations. Mid and high concentrations were within the standard bioanalytical acceptance of ±15 % [3] (Tables 3 to 5).

Table 3. Quality control A

Quality Control A (15.0 pg/mL)				
Laboratory	**A**	**B**	**C**	**D**
Rep 1	18.2	19.8	13.7	18.3
Rep 2	18.9	17.7	14.5	18.2
Rep 3	18.1	19.4	14.4	19.8
Rep 4	18.5	18.0	15.7	16.1
Rep 5	16.5	18.9	14.6	19.2
Rep 6	16.0	19.2	15.3	18.2
Mean	17.7	18.8	14.7	18.3
S.D.	1.2	0.8	0.7	1.3
% C.V.	6.6 %	4.3 %	4.8 %	6.9 %
% R.E.	21.3 %	25.3 %	-2.0	22.0 %
n	6	6	6	6

Table 4. Quality control B

Quality Control B (70.0 pg/mL)				
Laboratory	**A**	**B**	**C**	**D**
Rep 1	75.9	70.2	68.3	91.4
Rep 2	74.9	72.6	67.8	97.9
Rep 3	75.3	71.2	69.9	97.5
Rep 4	74.8	73.0	70.0	99.3
Rep 5	73.5	82.9	68.7	87.7
Rep 6	74.5	77.8	70.9	90.7
Mean	74.8	74.6	69.3	94.1
S.D.	0.8	4.8	1.2	4.8
% C.V.	1.1 %	6.5 %	1.7 %	5.0 %
% R.E.	6.9 %	6.6 %	-1.0 %	34.4 %
n	6	6	6	6

Table 5. Quality control C

Quality Control C (750 pg/mL)				
Laboratory	**A**	**B**	**C**	**D**
Rep 1	836	751	731	982
Rep 2	826	730	715	1060
Rep 3	813	770	752	959
Rep 4	805	664	769	936
Rep 5	823	769	759	990
Rep 6	831	783	745	933
Mean	822	745	745	977
S.D.	12	43	20	47
% C.V.	1.4 %	5.8 %	2.6 %	4.8 %
% R.E.	9.6 %	-0.7 %	-0.1 %	30.3 %
n	6	6	6	6

The use of the imbedded standard set prepared at the organizing laboratory allowed differentiation of biases observed from method-related issues and issues related to the reference materials used for standard calibration. Biases observed within the data for the low level quality control sample are significantly reduced with the shared standard calibrator set (Table 6 to Table 8). A single laboratory which reported a 22 to 34 % bias across all three levels of quality control samples showed reduced bias across all concentrations (-1.9 to 3.1 %).

Table 6. Quality control A

Quality Control A (15.0 pg/mL)				
Laboratory	**A**	**B**	**C**	**D**
Rep 1	16.6	17.0	13.7	13.5
Rep 2	17.2	15.0	14.5	15.8
Rep 3	16.5	16.7	14.4	15.1
Rep 4	16.9	15.3	15.7	15.1
Rep 5	15.1	16.1	14.6	15.1
Rep 6	14.7	16.4	15.3	16.2
Mean	16.2	16.1	14.7	15.1
S.D.	1.0	0.8	0.7	0.9
% C.V.	6.3 %	4.9 %	4.8 %	6.1 %
% R.E.	8.0 %	7.3 %	-2.0 %	0.7 %
n	6	6	6	6

Table 7. Quality control B

Quality Control B (70.0 pg/mL)				
Laboratory	**A**	**B**	**C**	**D**
Rep 1	69.0	64.5	68.3	76.0
Rep 2	68.1	66.7	67.8	67.5
Rep 3	68.5	65.4	69.9	69.9
Rep 4	68.0	64.5	70.0	69.8
Rep 5	66.8	66.7	68.7	75.3
Rep 6	67.7	65.4	70.9	74.4
Mean	68.0	65.5	69.3	72.2
S.D.	0.7	1.0	1.2	3.5
% C.V.	1.1 %	1.5 %	1.7 %	4.9 %
% R.E.	-2.9 %	-6.4 %	-1.0 %	3.1 %
n	6	6	6	6

Table 8. Quality control C (750 pg/mL)

Quality Control C (750 pg/mL)				
Laboratory	**A**	**B**	**C**	**D**
Rep 1	759	706	731	707
Rep 2	750	686	715	741
Rep 3	738	770	752	705
Rep 4	731	664	769	743
Rep 5	747	769	759	800
Rep 6	754	783	745	720
Mean	747	730	745	736
S.D.	10.4	50.6	19.6	35.3
% C.V.	1.4 %	6.9 %	2.6 %	4.8 %
% R.E.	-0.4 %	-2.7 %	-0.6 %	-1.9 %
n	6	6	6	6

Testing with the quality control samples prepared with the aglycone NNAL only clearly demonstrates that comparable results within standard bioanalytical acceptance levels are achievable between multiple laboratories. Differences in quantitation observed between laboratories could potentially be improved by consistent use of a well characterized reference material for the preparation of standard calibrators.

9. Total NNAL Result Comparison of NIST Urine Standard and Multiple Urine Lots

The validated methods employed by each laboratory are designed to measure total NNAL elimination in human urine. To achieve this, each method incorporates a hydrolysis step prior to the purification of the sample extract. The hydrolysis step must be effective in deconjugating both the N- and O-glucuronide metabolites of NNAL. The standard approach is to utilize a β-glucuronidase after pH adjustment of the urine aliquot. Each of the 4 assays included in this inter-laboratory comparison utilizes a version of the standard approach to complete the hydrolysis. The specific methods including the pH, source of β-glucuronidase, temperature and time of reaction were not requested from each laboratory.

To evaluate the consistency of quantitation between the laboratories, a set of urine samples was purchased. The purchased lots of urine were reported to be from both smoker and non-smoker volunteers. No details regarding product use were requested from the bio-repository. Additionally, a standard urine sample was purchased from NIST for use as a urine lot for testing. Including the NIST urine standard, a total of 10 urine lots were evaluated in triplicate to determine the comparability of the total NNAL reportable results.

The comparability of the unknown urine sample concentrations was performed by establishing a mean of all laboratory results and then applying an acceptance criterion of 20 % difference (% Diff), which is standard for incurred sample reproducibility testing [3]. This criterion is very broad considering the demonstrated precision of the assays seen in the above tables (1.1 to 6.9 % C.V.). However, as it is standardly used in regulated bioanalytical research for the analysis of small molecules it was applied.

The initial results provided in Tables 9 through 12 detail the sample results for each laboratory by lot number. The samples were quantitated with standard calibrators prepared by each laboratory.

Table 9. Lab A Total NNAL (pg/mL) for lots of smokers' urine

Lab A Total NNAL (pg/mL) for Lots of Smokers' Urine										
Replicate	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8	Lot 9	NIST Std
1	18.6	38.3	107	132	136	303	433	678	295	239
2	17.8	38	103	128	140	316	452	648	284	232
3	16.6	36.6	101	123	133	310	453	653	295	239
Mean	17.7	37.6	104	128	136	310	446	660	291	237
% Diff	-1.7 %	-4.2 %	2.9 %	0.8 %	-1.5 %	-0.3 %	1.8 %	-2.2 %	3.0 %	0.8 %

Table 10. Lab B Total NNAL (pg/mL) for lots of smokers' urine

Lab B Total NNAL (pg/mL) for Lots of Smoker Urine										
Replicate	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8	Lot 9	NIST Std
1	18.6	38.5	95.9	122	131	294	397	613	288	225
2	19.0	42.7	103	121	136	301	415	692	287	203
3	18.2	38.4	95.5	121	128	283	411	642	286	228
Mean	18.6	39.9	98	121	132	293	408	649	287	219
% Diff	3.3 %	1.6 %	-2.8 %	-4.6 %	-4.7 %	-6.0 %	-7.2 %	-4.0 %	-4.4 %	-7.0 %

Table 11. Lab C Total NNAL (pg/mL) for lots of smokers' urine

Lab C Total NNAL (pg/mL) for Lots of Smokers' Urine										
Replicate	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8	Lot 9	NIST Std
1	13.7	34.2	91.9	104	119	279	352	610	253	198
2	14.1	32.8	84.2	103	112	263	366	581	254	199
3	14.1	34.1	84.3	100	120	281	353	567	247	206
Mean	14.0	33.7	87	102	117	274	357	586	251	201
% Diff	-25 %	-15 %	-15 %	-22 %	-16 %	-13 %	-20 %	-14 %	-18 %	-16 %

Table 12. Lab D Total NNAL (pg/mL) for lots of smokers' urine

Lab D Total NNAL (pg/mL) for Lots of Smokers' Urine										
Replicate	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8	Lot 9	NIST Std
1	23.3	44.6	115	160	159	385	561	790	380	295
2	19.0	44.8	115	155	171	364	561	781	367	262
3	23.1	47.4	116	149	170	356	497	846	366	296
Mean	21.8	45.6	115	155	167	368	540	806	371	284
% Diff	19 %	15 %	13 %	20 %	19 %	17 %	21 %	18 %	21 %	19 %

When compared to the mean of all results, two laboratories demonstrated significant bias when the self-prepared standard calibrators are used for quantitation. Possible causes for the under estimation of total NNAL values could be due to incomplete hydrolysis. Possible causes for the over estimation of total NNAL values could be related to the use of a reference material with overestimated purity or poor selectivity. Quantitation of the same samples with the imbedded standard set can provide some clarity on the causes of the observed bias. This data is presented in Tables 13 through 16.

Table 13. Lab A Total NNAL (pg/mL) for lots of smokers' urine - Imbedded standard calibrators

Lab A Total NNAL (pg/mL) for Lots of Smoker Urine - Imbedded Standard Calibrators										
Replicate	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8	Lot 9	NIST Std
1	17.0	34.9	97.6	120	123	275	393	616	268	217
2	16.3	34.6	94.1	116	128	287	410	588	258	211
3	15.2	33.3	91.9	111	121	282	411	593	268	217
Mean	16.2	34.3	95	116	124	281	405	599	265	215
% Diff	1.7%	-1.8%	4.9%	3.2%	1.6%	1.6%	4.2%	-0.2%	-0.5%	3.3%

Table 14. Lab B Total NNAL (pg/mL) for lots of smokers' urine - Imbedded standard calibrators

Lab B Total NNAL (pg/mL) for Lots of Smokers' Urine - Imbedded Standard Calibrators										
Replicate	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8	Lot 9	NIST Std
1	15.9	34.6	88.6	113	122	274	372	575	270	210
2	16.3	38.5	95.7	112	126	282	389	649	269	175
3	15.5	34.5	88.2	112	119	265	385	603	268	213
Mean	15.9	35.9	91	112	122	274	382	609	269	199
% Diff	0.0 %	2.7 %	0.9 %	0.3 %	0.3 %	-1.2 %	-1.6 %	1.5 %	1.1 %	-4.4 %

Table 15. Lab C Total NNAL (pg/mL) for lots of smokers' urine - Imbedded standard calibrators

Lab C Total NNAL (pg/mL) for Lots of Smokers' Urine - Imbedded Standard Calibrators										
Replicate	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8	Lot 9	NIST Std
1	13.7	34.2	91.9	104	119	279	352	610	253	198
2	14.1	32.8	84.2	103	112	263	366	581	254	199
3	14.1	34.1	84.3	100	120	281	353	567	247	206
Mean	14.0	33.7	87	102	117	274	357	586	251	201
% Diff	-12.9 %	-3.5 %	-3.6 %	-9.0 %	-4.2 %	-1.0 %	-8.3 %	-2.4 %	-5.7 %	-3.4 %

Table 16. Lab D Total NNAL (pg/mL) for lots of smokers' urine - Imbedded cals

Lab D Total NNAL (pg/mL) for Lots of Smokers' Urine - Imbedded Cals										
Replicate	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8	Lot 9	NIST Std
1	18.9	34.9	87.6	121	120	292	425	594	288	223
2	15.6	35.0	87.5	118	130	275	423	588	277	199
3	18.7	37.0	88.2	114	129	269	374	637	276	223
Mean	17.7	35.6	88	118	126	279	407	606	280	215
% Diff	10.9 %	2.1 %	-2.5 %	4.9 %	3.5 %	0.6 %	4.9 %	1.1 %	5.2 %	3.3 %

With the use of a single standard calibrator set, the majority of the bias observed with the analysis of the lots of smoker urine was eliminated. All participating laboratories were successful in the quantitation of the 9 smoker urine samples as well as the NIST standard within 20 % difference. For all lots of urine with the exception of the lot with the lowest measured concentration, all mean results were within 10 % difference. Greater variability at approximately 3 times the lower limit in quantitation was observed for lot 1 (-12.9 to 10.0 % difference).

10. Conclusion

The inter-laboratory evaluation was completed successfully with 4 participating laboratories. The results demonstrate that the assays employed provide consistent results within standard bioanalytical acceptance criteria when a single source of reference material is used for quantitation. However an increased bias was observed when the standard calibrators separately prepared at each laboratory were used to perform the quantitation. As a reference the BMK Sub-Group has published a guideline on the characterization of reference standards [CORESTA Guide N° 20 - Biomarker Studies - Requirements for the Certification of Analytical Reference Standards].

11. Recommendations

After a review of the analytical results, three recommendations may be made. The first recommendation for future inter-laboratory comparison studies is to ensure a broad number of participating laboratories is identified. The strength of the inter-laboratory testing is significantly reduced if the number of participating laboratories is limited. Considerations should include ensuring the participating laboratories have the capability to receive samples through an international shipment prior to study initiation. Additionally, each participating laboratory should be able to indicate if the assay has been used within a reasonable period of time. It has been observed that an assay that has gone unused for an extended period of time may be problematic in bringing back into operation.

The second recommendation is that inquiries be sent to laboratories outside of those that participate in CORESTA to further expand the possible pool of participating laboratories. In this study an inquiry was successfully made to the University of Minnesota laboratory. The scientists at this laboratory are well published experts in TSNA analysis. As the BMK Sub-Group moves to test biomarkers of effect, a larger pool of laboratories with validated assays will be available to potentially participate. Every effort should be made to expand the list of participating laboratories to include those from industry, clinical research organizations, academia and government (CDC) laboratories if possible.

The final recommendation is that future studies include a similarly strong approach for inter-laboratory evaluation. The data from this study demonstrates that significant differences in the reported values could have been incorrectly attributed to analytical differences in the assays. With the inclusion of the imbedded standard, the data demonstrates that the participating laboratories could provide consistent results if a similarly qualified reference material were used by each laboratory. Without a well-qualified standard, differences in quantitation resulting in biases up to 40 % could be observed between laboratories.

12. Contributors

François Deschamps, Max Scherer, Nikola Pluym, Mark Bentley, Krishna Prasad, Kirk Newland, Steve Carmella, Eckhardt Schmidt and Gaddamanugu L. Prasad.

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