

Memo To: Robyn Weimer, Chemistry Program Manager
From: Rebecca Wagner, Ph.D., Chemistry Research Section Supervisor
CC: Alka Lohmann, Technical Services Director
Date: September 10, 2024
RE: Validation Plan: Quantitative Analysis of Fentanyl using Gas Chromatography-Flame Ionization Detection/Mass Spectrometry (GC-FID/MS)

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Validation Plan: Quantitative Analysis of Fentanyl using Gas Chromatography-Flame Ionization Detection/Mass Spectrometry (GC-FID/MS)

It is proposed to validate a method for the quantitative analysis of fentanyl using gas chromatography-flame ionization detection/mass spectrometry (GC-FID/MS). The Drug Enforcement Administration (DEA) Office of Forensic Sciences has provided the Virginia Department of Forensic Science with a Quantitative Method Validation Final Report for the Quantitation of Heroin Hydrochloride, Fentanyl Hydrochloride, and Fentanyl Citrate by Gas Chromatography (Method # DEA 127). In addition to the method validation, the instrumental parameters for the gas chromatography-flame ionization detection instrument were provided.

The instrumental method employs a hydrogen carrier gas with an Agilent Technologies HP-5 (12 m x 0.20 mm x 0.33 μ m film thickness) analytical column. The method uses a 1.0 μ L injection volume with a 270°C inlet temperature. The instrument is operated in split mode with a split ratio of 50:1. The hydrogen carrier gas flow rate was established at 1.5 mL/min. The oven temperature starts at 230°C for 1.0 minute with a ramp of 20°C/min to 300°C and a final hold of 0.5 min for a total run time of 5 minutes. The flame ionization detector is maintained at a temperature of 300°C.

The extraction solvent utilized within the DEA method (Method # DEA 127) employs 9:1 chloroform:methanol with 0.4 mg/mL tetracosane internal standard. Samples are weighed and dissolved in internal standard solution. The solution is filtered using glass wool prior to analysis if an appreciable amount of insoluble material is present. An additional sample preparation method using a basic extraction was also included in the DEA method validation. The basic extraction is performed by adding 1.0 - 3.0 mL of the prepared solution into a test tube. A 1.0-3.0 mL aliquot of 2 N sodium hydroxide (NaOH) is then added to the test tube and mixed thoroughly. The sample is then centrifuged, and the chloroform layer (bottom) is removed for analysis.

During the initial assessment of the DEA method, a change in internal standard response from a low concentration to a high concentration was noted. The change in internal standard response was significant and caused a bias within the calibration range outside of the acceptance criteria of $\pm 5\%$. An investigation into the source of bias was performed and noted to be an impact of the amount of methanol added into the calibrators. The elimination of methanol as a component of the extraction solvent corrected the bias observed with a 9:1 chloroform:methanol extraction solvent. Due to the change in extraction solvent, a validation will be performed comparing both extraction solvents. The additional experiments will demonstrate if the change in extraction

solvent negatively affects the analytical result. Additional experiments delineated within the validation plan include the assessment of the calibration model, dilution integrity, and recovery. This will be performed with and without base extraction in addition to evaluating the two different extraction solvents.

Within the DEA 127 method, several different ranges were proposed for the working range. The working range of the method for fentanyl hydrochloride was 0.0308 mg/mL to 1.541 mg/mL. The working range of the method for fentanyl citrate quantitation was 0.250 mg/mL to 1.393 mg/mL. With the base extraction step, the working range of the method was 0.129 mg/mL to 1.676 mg/mL. The working range was established based on the concentrations evaluated for accuracy and not the originally proposed working range of the method. The originally proposed working range of the method was 0.0296 mg/mL to 1.482 mg/mL based on the preparation of calibrators. A total of 10 calibrators were utilized during the original validation with a linear regression model selected as the best fit calibration model. The limit of quantitation was determined to be 0.0776 mg/mL. Selectivity, linearity, lower limit of quantitation, repeatability, and accuracy (bias) were evaluated during the DEA validation.

The DEA validated linear range of the base extraction sample preparation was slightly different than the non-base extracted method. For the base extraction, the originally proposed validated linear range from the DEA method was 0.0303 mg/mL to 1.515 mg/mL.

A verification plan is outlined herein pursuant to the Quality Manual (Qualtrax Revision 28) and Controlled Substances Procedures Manual (Qualtrax Revision 22).

1. Accuracy (Bias) and Precision
2. Linearity/Calibration Model
3. Lower Limit of Quantitation (LLOQ)
4. Selectivity
5. Repeatability
6. Recovery
7. Dilution Integrity
8. Carryover
9. References

Validation experiments will be performed on an Agilent Technologies 8890 gas chromatograph coupled with a flame ionization detector and a 5977C mass spectrometer. The instrumental parameters from the DEA 127 method will be followed, as applicable. Once established, the parameters will not be modified during verification.

1. Accuracy (Bias)

The bias of the method will be verified using fentanyl HCl and fentanyl citrate as described in the DEA 127 method validation. The following samples will be prepared as described in the DEA 127 method including: Recovery #2, Recovery #3, Recovery #4, Recovery #5, Recovery #8, and Recovery #10. The preparation of these samples will be of similar concentration and purity. The masses and volumes may be different. Recovery #3 will also include quinine to compensate for the mass of heroin utilized. In Recovery #4 lactose and inositol will replace ANPP, hydroxyzine, and p-fluorofentanyl which are evaluated during selectivity. Three injections of each sample will be analyzed with and without base extraction. The bias shall be within $\pm 5\%$ for each sample.

2. Linearity/Calibration Model

The linearity of the DEA 127 method was established using 10 calibrators. The calibrator concentrations utilized are delineated in Table 1. The actual concentration is the concentration of each calibration level corrected for the purity and mass of the standard.

Table 1 DEA 127 method calibrator concentrations

DEA 127 Method Calibrators	
Calibrator Level	Actual Concentration (mg/mL)
1	1.481687
2	1.333518
3	0.889012
4	0.740843
5	0.592672
6	0.296337
7	0.148168
8	0.088901
9	0.059267
10	0.029633

During method validation, all 10 calibrators will be prepared and analyzed a minimum of five times to establish the regression model. Residual analysis and statistical comparisons (ANOVA) will be utilized to establish the best fit calibration model. A plot of the residual values for each calibration type shall be generated to evaluate the effectiveness of the calibration model. The plot(s) will be visually evaluated to determine the model with homoscedasticity over the working range. Fentanyl without base extraction and with base extraction sample preparation will be evaluated for linearity using the 10 calibration levels.

The DEA 127 method utilized different calibrator concentrations when performing the fentanyl base extraction sample preparation. Additionally, 11 calibrators were used as opposed to 10

calibrators used in the evaluation without base extraction. These concentrations are delineated in Table 2.

Table 2 DEA 127 method calibrator concentration with base extraction

DEA 127 Method Calibrators	
Calibrator Level	Actual Concentration (mg/mL)
1	1.36375
2	1.21223
3	1.06070
4	0.90917
5	0.75764
6	0.60611
7	0.36367
8	0.24245
9	0.12122
10	0.06061
11	0.03031

The validation of fentanyl with base extraction sample preparation will be performed using the same calibration levels delineated in Table 1. During method validation, all 10 calibrators will be prepared and analyzed a minimum of five times to establish the regression model for the base extraction sample preparation. Residual analysis and statistical comparisons (ANOVA) will be utilized to establish the best fit calibration model. A plot of the residual values for each calibration type shall be generated to evaluate the effectiveness of the calibration model. The plot(s) will be visually evaluated to determine the model with homoscedasticity over the working range. An R² of 0.99996 was established for fentanyl HCl without base extraction during method validation. The base extracted calibration with 11 calibrators had a R² of 0.99988. At a minimum, the correlation coefficient, R², for each calibration curve (base extracted and non-base extracted) shall be ≥0.995.

3. Lower Limit of Quantitation (LLOQ)

The lower limit of quantitation will be verified using the data obtained from the linearity evaluation. The linear regression equation will be determined for each calibration curve and utilized in the determination of the lower limit of quantitation. The lower limit of quantitation will be calculated using Equation 1.

Equation 1

$$LLOQ = \frac{10\sigma}{m}$$

Where σ is the standard deviation of the y-intercept and m is the slope of the linearity function. The calculated lower limit of quantitation for fentanyl with and without base extraction shall be ≤ 0.0776 mg/mL as established during method validation for fentanyl with base extraction.

4. Selectivity

To evaluate interferences from commonly encountered drugs and bulking agents, reference materials prepared in extraction solvent will be analyzed without internal standard (tetracosane). A single injection of each compound will be analyzed with and without base extraction. To establish an interferent database, all compounds evaluated within the DEA 127 method validation and additional compounds, as appropriate, will be evaluated. The retention time and relative retention time from fentanyl and tetracosane will be evaluated.

5. Repeatability

Repeatability was evaluated during the DEA 127 method validation by assessing three different calibrator concentrations without base extraction. The calibrators evaluated were 1.481687 mg/mL, 0.740843 mg/mL, and 0.029633 mg/mL. During the evaluation of repeatability with the base extraction sample preparation, the undiluted standard (1.51528 mg/mL) and the lowest calibrator (0.03031 mg/mL) were evaluated. The validation will assess the variability of the 1.481687 mg/mL, 0.740843 mg/mL, and 0.029633 mg/mL calibrators with and without base extraction. The average, standard deviation, and percent relative standard deviation will be calculated for each calibration level. Table 3 lists the percent relative standard deviation for each calibration level evaluated.

Table 3 Repeatability percent relative standard deviation

DEA 127 Method Calibrators	
Concentration (mg/mL)	%RSD
1.481687	0.6787
0.740843	0.0410
0.029633	0.3830

It was also noted that the DEA 127 method validation had a repeatability acceptance criterion of a percent relative standard deviation of less than 2%. For acceptance, the percent relative standard deviation shall be within 2% but will also be compared to the values obtained from the data within the method validation.

6. Recovery

The recovery of the method will be assessed by comparing the instrumental response from extracted samples that contain fentanyl as well as common diluents to a neat fentanyl sample. The ratioed instrumental response of analyte to internal standard will be evaluated and

compared between the extracted sample and neat standard analysis. The deviation of the extracted ratioed response shall be within $\pm 2\%$ of the neat fentanyl standard ratioed response. The recovery will be evaluated at a low and high concentration of fentanyl, with triplicate analysis per concentration.

7. Dilution Integrity

Dilution integrity will be assessed using the data obtained from the bias evaluation. Each sample prepared will be analyzed undiluted as well as with several dilution factors. The diluted concentrations will be compared to the undiluted concentrations for an assessment of dilution integrity. The bias from diluted samples shall be within $\pm 2\%$ of the undiluted concentration.

8. Carryover

Carryover will be evaluated with solvent blanks injected immediately following the highest calibrator concentration (1.48 mg/mL) and after each accuracy (bias) and repeatability sample. The solvent blanks are deemed free from carryover when no instrumental response is noted in the solvent blanks.

9. References

Virginia Department of Forensic Science Quality Manual, (Qualtrax Revision 28). 2024.

Virginia Department of Forensic Science Controlled Substances Procedure Manual (Qualtrax Revision 22). 2024.

Drug Enforcement Administration Office of Forensic Sciences Quantitative Method Validation Final Report – Level 1. Quantitation of Heroin Hydrochloride, Fentanyl Hydrochloride, and Fentanyl Citrate by Gas Chromatography, Method # DEA 127. 07/24/2023.

Drug Enforcement Administration Office of Forensic Sciences Method Information. DEA127_B.M. Modified 2/10/2017.