Validation Report for Quantitation and/or Qualitative Identification of Benzodiazepines, Opioids, and Select Hypnotics in Whole Blood by LC-MSMS

This document describes the validation of benzodiazepines, opioids, and select hypnotics for quantitation and/or qualitative identification by liquid chromatography with tandem mass spectrometry (LC-MSMS). Protein precipitation with acetonitrile was used to prepare the whole blood specimens for analysis. A Shimadzu Prominence liquid chromatograph with an AB Sciex 3200 QTrap tandem mass spectrometer, designated as LC-1, was used for quantitation and confirmation using multiple reaction monitoring mode (MRM). Dihydrocodeine and flunitrazepam were validated for qualitative identification only. Buprenorphine, norbuprenorphine, and ramelteon were not able to be validated for qualitative or quantitative identification by this method. This validation included the evaluation of:

| **Validation Parameter** | **Acceptance Criteria (1-5)** | **Validation Results** |
|---|---|---|
| Selectivity/Specificity | 1. No matrix interference from at least 10 different whole blood sources that do not contain the target analyte(s).<br>2. No interference from at least 10-15 similar compounds that are commonly identified in whole blood case samples.<br>3. No interference from a high concentration of target compound(s) for the internal standard(s). | 1. There was no matrix interference from 10 different whole blood sources that did not contain the target analytes.<br>2. There was no interference from 53 similar compounds that are commonly identified in whole blood case samples.<br>3. There was no significant interference from a high concentration of target compounds for the internal standards. |
| Ionization Suppression/Enhancement | 1. Isotopically-labeled (deuterium) internal standards that co-elute (within ± 0.05 minutes) with each target analyte will be used for all but one compound. Therefore, ionization suppression/enhancement experiments are not required or necessary except for that one compound. However, SWGTOX (5) lists this experiment as a requirement and it will therefore be conducted.<br>2. Average suppression or enhancement must be less than ± 25% and the %CV of the suppression or enhancement must be less than 15%. If either of these values are exceeded then it must be demonstrated that the | 1. Several compounds demonstrated average suppression or enhancement greater than ± 25% for either the analyte, internal standard or both. None of the compounds demonstrated average suppression or enhancement greater than ± 11% for the relative response indicating that the use of isotopically-labeled internal standards compensated for any significant ion suppression or enhancement.<br>2. The % CV for the relative response was not greater than 10% for any compound. |

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| | suppression/enhancement does not impact LOD, LOQ, and bias. | |
| Calibration model/linearity | 1. At least 6 non-zero concentrations evenly spaced across the calibration range (perhaps more for non-linear models), with five replicates at each level analyzed in 5 separate extractions (one replicate per level per extraction), with the combined data used to establish the calibration model.<br>2. Coefficient of determination must be ≥ 0.990.<br>3. Visual inspection of the curve and residual plot should indicate normal random scatter around the calibration curve.<br>4. Calibrators must be within ± 20% of their prepared concentration. | 1. At least six non-zero concentrations over 5 separate extractions were used to establish the calibration model.<br>2. The coefficient of determination ($r^2$) was ≥ 0.990 for all compounds except chlordiazepoxide. A more appropriate minimum coefficient of determination for chlordiazepoxide will be specified in the method as ≥ 0.980.<br>3. Visual inspection of the curves and residual plots indicated normal random scatter around the calibration curve.<br>4. All calibrators were within ± 20% of their prepared concentration. |
| Sensitivity – Limit of Detection (LOD) | 1. Good chromatographic peak shape.<br>2. Signal-to-noise ratio of greater than 3:1.<br>3. MRM ion ratios within 20% and retention time within 0.1 minutes (or 2% relative retention time) compared to a suitable standard (or average of all calibrators).<br>4. Once determined, the LOD must be verified by at least two replicates of a standard run in three separate extractions prepared in three different sources of blood. | 1. All compounds had good chromatographic peak shape at the LOD.<br>2. All signal-to-noise ratios were greater than 20:1.<br>3. MRM ion ratios were within 20% and retention times were within 0.1 minutes compared to the average of all calibrators used (specific to each compound).<br>4. The validated LOD was verified by at least two replicates of a standard run in five separate extractions prepared in whole blood containing a mixture of four different sources. |
| Sensitivity—Limit of Quantitation (LOQ) | 1. Good chromatographic peak shape.<br>2. Signal to Noise ratio of greater than 10:1.<br>3. MRM Ion ratios within 20% and retention time within 0.1 minutes (or 2% relative retention time) compared to a suitable standard (or average of all calibrators).<br>4. Quantitative results must be within ± 20% of their prepared concentration.<br>5. Once determined, the LOQ must be | 1. All compounds had good chromatographic peak shape at the LOQ.<br>2. All signal to noise ratios were greater than 31:1 for those compounds validated for quantitative analysis.<br>3. MRM ion ratios were within ± 20% and retention times were within 0.1 minutes compared to the average of all calibrators used (specific to each compound). |

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| | verified by at least two replicates of a standard run in three separate extractions prepared in three different sources of blood. | 4. All quantitative results were within ± 20% of their prepared concentration.<br>5. The LOQ was verified by two replicates of a standard run in five separate extractions prepared in a mixture of whole blood containing four different sources. |
| Repeatability—Bias (Accuracy) | 1. Evaluated at three concentration levels. A low level within three times the LOQ, a high level within 20% of the upper limit of the calibration range, and a medium level near the midpoint of the low and high. Pooled fortified matrix samples may not be used as some target analytes may demonstrate poor stability in matrix.<br>2. At least 3 replicates from 5 separate extractions should be evaluated for each level.<br>3. The bias should be within ± 20% of the prepared concentration. | 1. Standards prepared at least at 3 different concentrations—low, medium, and high level meeting validation requirements—were evaluated for each compound.<br>2. Three replicates from five separate extractions were evaluated for each level.<br>3. The bias was within ± 20% of the prepared concentration. |
| Repeatability—Precision<br>Within-run<br>Between-run | 1. Evaluated at least at three concentration levels. The same that were used for the bias studies.<br>2. At least 3 replicates from 5 separate extractions should be evaluated for each level.<br>3. The coefficient of variation (CV) should be within 20%. | 1. At least three levels were evaluated.<br>2. Three replicates from five separate extractions were evaluated for each level.<br>3. The CV was within 20%. |
| Reportable Range | 1. The reportable range shall be determined after evaluating the calibration model and sensitivity of the assay.<br>2. It is advantageous, but may not be necessary for the reportable range to include the range of desired concentrations noted below. | 1. The reportable range was determined for each compound. |
| Dilution Integrity | 1. Any required dilutions of case samples will be made with whole blood. Dilution integrity will be evaluated at a 1:10 dilution by repeating bias and precision studies at one level using a 1:10 dilution of standards prepared in whole blood. Other dilutions may also be | 1. Dilution integrity was evaluated for a 1:10 dilution of a 200/2000 ng/mL control prepared in whole blood.<br>2. All stated bias and precision criteria were acceptable using the dilution. |

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| | evaluated if it is anticipated that they will be routinely used in casework.<br>2. All bias and precision criteria stated above must be acceptable when using the dilution. | |
| Carryover | 1. A blank matrix sample must be analyte free when run after a standard prepared at or above the highest calibrator concentration.<br>2. Lack of carryover must be determined by triplicate analyses (repeated injection of an extracted standard and blank is acceptable). | 1. The blank matrix sample was analyte free when run after a standard prepared at 100/1000 ng/mL.<br>2. The lack of carryover was determined by triplicate analyses on five different days. |
| Extract Stability | 1. Five replicates of controls will be prepared at a low and high concentration. The extracts will be combined and then divided into five different vials. A vial of each level will be injected in triplicate on day 0. The other vials will be stored on the instrument and re-injected on each subsequent day in triplicate.<br>2. The response of each analyte must be within ± 20% of the response from day 0. If the response falls outside this range then the extract stability of the analyte has been exceeded. | 1. Extract stability varied from 0 to 4 days depending on the drug. The extract stability of many drugs was exceeded on day 1. It is recommended that extracts not be rerun in excess of 24 hours after extraction unless the rerun is for a drug that demonstrated suitable extract stability. |
| Ruggedness/Robustness | 1. Validation studies will be performed over multiple days by multiple analysts. | 1. Studies were performed by 5 different analysts over multiple days and demonstrated repeatable results. |
| Case Sample Comparison | 1. Any case samples that have been previously determined to contain the target analyte(s) must have identical qualitative results and quantitative results must agree within ± 20% (within the capabilities of the methods being compared). Note: Some analytes may have poor stability in matrix and this should be considered if there are discrepancies in the qualitative and/or quantitative results. | 1. Overall good agreement for case samples that had been previously determined to contain the target analytes was observed. Qualitative results were consistent within the capabilities of each method. Quantitative results for most target analytes were within ± 20%. Some discrepancies were noted and are discussed below. |

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| Estimation of Uncertainty of Measurement | 1. The uncertainty of measurement estimation worksheet will be constructed using TX Estimation of Uncertainty of Measurement (UOM) for the replicate data of a suitable control that will be used in routine casework. Note: This requires a minimum of 30 replicates. | 1. The uncertainty of measurement was estimated using a minimum of 30 replicates of the 20/200 ng/mL control that will be used for routine casework using TX Estimation of Uncertainty of Measurement (UOM). |

# Validation Steps

**Step 1:** **Ionization Suppression/Enhancement**
12/30/13
Analyst: Nick Tiscione (NBT)

General Outline of Validation Step

1. Prepare working calibrator(s) and control(s) in acetonitrile or other suitable solvent.
2. Prepare working internal standard in acetonitrile or other suitable solvent.
3. Two different sets of samples will be prepared and the analyte and internal standard peak areas of neat standards will be compared to matrix samples fortified with neat standards after extraction or processing.
4. Set one will consist of neat standards prepared at two concentrations—one low and one high with one replicate at each level. Each of these neat standards will be injected six times to establish a mean peak area for each concentration.
5. Set two will consist of a minimum of ten different matrix sources (if possible). Each matrix source will be extracted in duplicate. After the extraction is complete, each matrix sample will then be fortified with either the low or high concentration neat standard.
6. The average area of each set ($\bar{X}$) is used to estimate the suppression/enhancement effect at each concentration as follows:

$$Ionization\ suppression\ or\ enhancement\ (\%) = \left(\frac{\bar{X}\ Area\ of\ Set\ 2}{\bar{X}\ Area\ of\ Set\ 1} - 1\right) x\ 100$$

**Step 2:** **Sensitivity, Carryover, Linearity, and Selectivity/Specificity**
1/3/14
Analyst: NBT

General Outline of Validation Step

1. Extract the following:
    a. A series of at least eight calibrators prepared in whole blood representing anticipated concentrations in whole blood specimens, evenly spaced. Two different desired calibration ranges will be evaluated, a low and a high.
    b. The low concentration range will include the following compounds:
        1. 6-MAM
        2. 7-Aminoflunitrazepam
        3. Buprenorphine
        4. Flunitrazepam

5. Fentanyl
6. Hydromorphone
7. Norbuprenorphine
8. Oxymorphone
9. Ramelteon

c. The high concentration range will include the following compounds:
    1. Alprazolam
    2. Chlordiazepoxide
    3. Clonazepam
    4. Codeine
    5. Desalkylflurazepam
    6. Diazepam
    7. Dihydrocodeine
    8. Hydrocodone
    9. Lorazepam
    10. Methadone
    11. Midazolam
    12. Morphine
    13. Nordiazepam
    14. Oxazepam
    15. Oxycodone
    16. Temazepam
    17. Zaleplon
    18. Zolpidem
    19. Zopiclone

| *Concentration range* | *Desired Range (ng/mL)* | *Suggested Calibrator Levels (ng/mL)* |
|---|---|---|
| Low | 1-50 | 0.1, 0.5, 1, 3, 5, 10, 20, 30, 40, 50, 100 |
| High | 10-500 | 1, 5, 10, 30, 50, 100, 200, 300, 400, 500, 1000 |

d. A matrix blank with internal standard to be run after the highest calibrator (analyzed in triplicate).
e. A matrix blank fortified with the target analytes at the same concentration as the highest calibrator without internal standard.
f. A matrix blank fortified with as many of the following compounds as are available, each at 10 ug/mL. Do not add internal standard. Note: Add appropriate amount of each compound to labeled tube, evaporate solvent and add whole blood.
    1. Acetaminophen
    2. Amitriptyline
    3. Amphetamine
    4. Benzoylecgonine
    5. Bupropion
    6. Butalbital
    7. Butylone
    8. BZP
    9. Carbamazepine
    10. Carisoprodol
    11. Chlorophenylpiperazine
    12. Chlorpheniramine
    13. Citalopram
    14. Cocaethylene
    15. Cocaine
    16. Cyclobenzaprine

17. Dextromethorphan
18. Delta-9 THC
19. Delta-9-Carboxy THC
20. Dicyclomine
21. Diphenhydramine
22. Doxepin
23. Doxylamine
24. Fluoxetine
25. 5-MeO-Dipt (Foxy)
26. Hydroxyzine
27. Lamotrigine
28. Lidocaine
29. Meperidine
30. Meprobamate
31. Metaxalone
32. Methamphetamine
33. Methylone
34. Metoclopramide
35. Mirtazepine
36. MDA
37. MDMA
38. Naloxone
39. Naltrexone
40. Norfluoxetine
41. Norpropoxyphene
42. Norquetiapine
43. Nortriptyline
44. Olanzapine
45. Orphenadrine
46. Pheniramine
47. Phenobarbital
48. Phentermine
49. Phenytoin
50. Promethazine
51. Propoxyphene
52. Pseudoephedrine
53. Sertraline
54. Topiramate
55. TFMPP
56. Tramadol
57. Trazodone
58. Venlafaxine
59. Quetiapine

g. Ten whole blood blanks from different sources that do not contain the target analytes or internal standard. Note: A blood blank that consists of a mixture of different sources may substitute for some of the ten (i.e. a mixture of six sources will count as six of the required ten).

2. Analyze on LC-1.
3. Repeat 1.a. through 1.d. four times in four separate extractions to yield 5 replicates at each calibrator level.
4. Use the combined data to evaluate the calibration model.
5. Evaluate the calibrators to determine the limit of detection (LOD) and limit of quantitation (LOQ).
6. Determine suitable levels for the calibrators to be used in routine analysis (at least 4 for linear models or at least 6 for non-linear models).

7. Determine suitable level(s) for the positive control(s) to be used in routine analysis.
8. Evaluate carryover and specificity.

**Steps 3-7:** **Sensitivity, Repeatability, Robustness, Estimation of Uncertainty of Measurement**

12/31/13 – 1/29/14
Analysts: NBT, Tate Yeatman (DTY), Amber Kohl (AK), Xiaoqin Shan (XS), Ilene Alford (IKA)

General Outline of Validation Steps

1. Prepare the calibrators, matrix blank, and replicates for each of the positive control(s).
    a. Positive controls
        i. Two replicates at the LOD prepared in at least three different sources or a mixture of at least three different sources of whole blood.
        ii. Two replicates at the LOQ (if different than LOD) prepared in at least three different sources or a mixture of at least three different sources of whole blood.
        iii. Three replicates at a low level within three times the LOQ.
        iv.
        v. Three replicates at a high level within 20% of the upper limit of the calibration range.
        vi. Three replicates at a medium/mid level near the midpoint of the low and high.
        vii. Three replicates of a standard prepared at ten times the concentration of the mid level, then diluted 1:10 with blank whole blood.
2. Analyze on LC-1, running the matrix blank after the highest calibrator.
3. Evaluate the positive controls for precision and accuracy (bias).
4. A suitable control level that will be analyzed for routine analysis will be used to establish an initial estimation of the UOM for the compound(s) being validated for quantitative analysis. See Step 9.

**Step 8:** **Case Sample Comparison/Evaluation**

Analysts: NBT, IKA, DTY, AK, XS

General Outline of Validation Step

1. Prepare the calibrators, matrix blank and positive control(s).
2. Prepare at least 5-10 negative and positive cases (as many as available).
3. Perform procedure and run on LC-1.

**Step 9:** **Estimation of Uncertainty of Measurement**

2/12/14
Analyst: NBT

General Outline of Validation Step

1. Prepare the calibrators and matrix blank.
2. Prepare enough replicates of the positive control to be used for the UOM estimation (identified in Steps 3-7) to yield a total of 30 when combined with replicates from Steps 3-8.
3. Perform procedure and run on LC-1.
4. Use TX Estimation of Uncertainty of Measurement (UOM) to establish an initial estimation of the UOM for the compound(s) being validated for quantitative analysis.

**Step 10:** **Extract Stability**
1/13/14
Analyst: NBT

General Outline of Validation Step

1. Prepare five replicates of controls at a low, mid and high concentration (same as used above in Steps 3-7) with internal standard.
2. Combine the extracts of the replicates at each level and then divide into five different vials.
3. Inject a vial of each level in triplicate on day 0.
4. Store the other vials on the instrument and reinject on each subsequent day in triplicate.
5. The response of each analyte must be within ± 20% of the response from day 0. If the response falls outside this range then the extract stability of the analyte has been exceeded.

# Results

**Selectivity / Specificity**

Several different blood samples were prepared and extracted to evaluate the selectivity of the method through an interference study. The specific samples are outlined below.

- A matrix blank fortified with the target analytes at the same concentration as the highest calibrator (100 or 1000 ng/mL depending on the analyte) without internal standard.
- A matrix blank fortified with 53 related compounds that have been identified in blood drug analysis casework. Each compound was fortified at a concentration of 10 µg/mL.
- Ten whole blood samples from different sources that did not contain the target analytes or internal standard.

There was no matrix interference from 10 different whole blood sources that did not contain the target analytes. There was no interference from 53 related compounds that are commonly identified in whole blood case samples. There was no significant interference from a high concentration of target compounds for the internal standards, although some interference was observed for codeine-$d_3$ from codeine fortified at 1000 ng/mL. The response observed was approximately 16% of the normal codeine-$d_3$ response and did not affect the accurate quantitative measurement of controls prepared at 5 levels across the calibration range of 10 to 500 ng/mL.

*Conclusion*

The method is specific for the target analytes and internal standards studied.

**Ionization Suppression/Enhancement**

Working standards for the calibrators, controls, and internal standard were prepared in acetonitrile. Two different sets of samples were prepared and the analyte and internal standard peak areas of neat standards were compared to matrix samples fortified with neat standards after extraction or processing. Set one consisted of neat standards prepared at two concentrations 5/50 ng/mL and 40/400 ng/mL with one replicate at each level. Each of the neat standards was injected six times to establish a mean peak area for each concentration. Set two consisted of ten different matrix sources that were extracted in duplicate. After the extraction was complete each matrix sample was fortified with the low or high concentration neat standard. The average area of each set was used to estimate the suppression/enhancement effect at each concentration as follows for the analyte, internal standard, and relative response for each drug:

$$Ionization\ suppression\ or\ enhancement\ (\%) = \left(\frac{\bar{X}\ Area\ of\ Set\ 2}{\bar{X}\ Area\ of\ Set\ 1} - 1\right) x\ 100$$

The % CV was also calculated for the analyte, internal standard, and relative response for each drug at each concentration.

| Analyte | ISTD | Levels Evaluated (ng/mL) | Ionization Suppression or Enhancement (%) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 5/50 ng/mL | | | 40/400 ng/mL | | |
| | | | Analyte Response | ISTD Response | Relative Response | Analyte Response | ISTD Response | Relative Response |
| 6-mam 1 | IS 6-mam d3 | 5, 40 | 27% | 22% | 3% | 6% | 3% | 3% |
| 7-aminoflunitrazepam 1 | IS 7-aminoflunitrazepam d7 | 5, 40 | 25% | 12% | 11% | 7% | -1% | 8% |
| alprazolam 1 | IS alprazolam d5 | 50, 400 | 50% | 41% | 6% | 10% | 11% | -1% |
| buprenorphine 1 | IS buprenorphine d4 | 5, 40 | 47% | 41% | 5% | 3% | 15% | -10% |
| chlordiazepoxide 1 | IS chlordiazepoxide d5 | 50, 400 | 8% | 5% | 3% | -6% | -6% | 0% |
| clonazepam 1 | IS clonazepam d4 | 50, 400 | 8% | 8% | 0% | -1% | -5% | 4% |
| codeine 1 | IS codiene d3 | 50, 400 | 14% | 14% | 0% | 0% | 1% | -1% |
| desalkylflurazepam 1 | IS desalkyflurazepam d4 | 50, 400 | 10% | 7% | 3% | -3% | -1% | -3% |
| diazepam 1 | IS diazepam d5 | 50, 400 | 9% | 10% | -1% | -7% | -6% | -1% |
| dihydrocodeine 1 | IS dihydrocodeine d6 | 50, 400 | 16% | 12% | 4% | -1% | -2% | 0% |
| fentanyl 1 | IS fentanyl d5 | 5, 40 | 16% | 16% | 0% | -2% | -1% | -1% |
| flunitrazepam 1 | IS flunitrazepam d7 | 5, 40 | 10% | 10% | 1% | -7% | -7% | 0% |
| hydrocodone 1 | IS hydrocodone d3 | 50, 400 | 3% | 6% | -2% | -7% | -10% | 3% |
| hydromorphone 1 | IS hydromorphone d3 | 5, 40 | -1% | -2% | 1% | -10% | -10% | 0% |
| lorazepam 1 | IS lorazepam d4 | 50, 400 | 14% | 3% | 11% | -11% | -8% | -2% |
| methadone 1 | IS methadone d3 | 50, 400 | 12% | 8% | 3% | -22% | -22% | 1% |
| midazolam 1 | IS midazolam d4 | 50, 400 | 21% | 25% | -3% | 3% | 6% | -3% |
| morphine 1 | IS morphine d3 | 50, 400 | 10% | 6% | 3% | -5% | -6% | 1% |
| norbuprenorphine 1 | IS norbuprenorphine d3 | 5, 40 | 18% | 22% | -4% | 1% | 3% | -3% |
| nordiazepam 1 | IS nordiazepam d5 | 50, 400 | 10% | 15% | -4% | -5% | -4% | -1% |
| oxazepam 1 | IS oxazepam d5 | 50, 400 | -5% | -8% | 4% | -11% | -13% | 3% |
| oxycodone 1 | IS oxycodone d3 | 50, 400 | 11% | 14% | -2% | -5% | -3% | -2% |
| oxymorphone 1 | IS oxymorphone d3 | 5, 40 | 2% | 1% | 1% | -9% | -5% | -4% |
| ramelteon 1 | IS alprazolam d5 | 5, 40 | 46% | 41% | 5% | 5% | 11% | -5% |
| temazepam 1 | IS temazepam d5 | 50, 400 | 16% | 23% | -6% | -6% | 3% | -8% |
| zaleplon 1 | IS zaleplon d4 | 50, 400 | 29% | 30% | -3% | -2% | -2% | 0% |
| zolpidem 1 | IS zolpidem d6 | 50, 400 | 8% | 8% | 0% | -2% | -2% | -1% |
| zopiclone 1 | IS zopiclone d4 | 50, 400 | 16% | 14% | 1% | 0% | -4% | 4% |

| Analyte | ISTD | Levels Evaluated (ng/mL) | % CV | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 5/50 ng/mL | | | 40/400 ng/mL | | |
| | | | Analyte Response | ISTD Response | Relative Response | Analyte Response | ISTD Response | Relative Response |
| 6-mam 1 | IS 6-mam d3 | 5, 40 | 7% | 3% | 9% | 5% | 4% | 4% |
| 7-aminoflunitrazepam 1 | IS 7-aminoflunitrazepam d7 | 5, 40 | 9% | 11% | 6% | 6% | 8% | 3% |
| alprazolam 1 | IS alprazolam d5 | 50, 400 | 8% | 7% | 5% | 10% | 7% | 4% |
| buprenorphine 1 | IS buprenorphine d4 | 5, 40 | 8% | 4% | 8% | 6% | 2% | 6% |
| chlordiazepoxide 1 | IS chlordiazepoxide d5 | 50, 400 | 5% | 4% | 3% | 2% | 3% | 3% |
| clonazepam 1 | IS clonazepam d4 | 50, 400 | 13% | 10% | 5% | 9% | 6% | 7% |
| codeine 1 | IS codiene d3 | 50, 400 | 8% | 8% | 3% | 6% | 6% | 2% |
| desalkylflurazepam 1 | IS desalkyflurazepam d4 | 50, 400 | 14% | 14% | 5% | 7% | 7% | 3% |
| diazepam 1 | IS diazepam d5 | 50, 400 | 8% | 7% | 4% | 5% | 5% | 3% |
| dihydrocodeine 1 | IS dihydrocodeine d6 | 50, 400 | 4% | 3% | 6% | 4% | 4% | 3% |
| fentanyl 1 | IS fentanyl d5 | 5, 40 | 6% | 5% | 3% | 4% | 4% | 2% |
| flunitrazepam 1 | IS flunitrazepam d7 | 5, 40 | 9% | 8% | 8% | 5% | 6% | 6% |
| hydrocodone 1 | IS hydrocodone d3 | 50, 400 | 8% | 7% | 3% | 6% | 7% | 2% |
| hydromorphone 1 | IS hydromorphone d3 | 5, 40 | 8% | 5% | 5% | 3% | 5% | 5% |
| lorazepam 1 | IS lorazepam d4 | 50, 400 | 15% | 11% | 8% | 7% | 8% | 6% |
| methadone 1 | IS methadone d3 | 50, 400 | 21% | 17% | 8% | 17% | 21% | 6% |
| midazolam 1 | IS midazolam d4 | 50, 400 | 6% | 6% | 2% | 2% | 4% | 3% |
| morphine 1 | IS morphine d3 | 50, 400 | 10% | 10% | 4% | 9% | 9% | 3% |
| norbuprenorphine 1 | IS norbuprenorphine d3 | 5, 40 | 8% | 7% | 3% | 8% | 6% | 6% |
| nordiazepam 1 | IS nordiazepam d5 | 50, 400 | 11% | 10% | 4% | 3% | 4% | 3% |
| oxazepam 1 | IS oxazepam d5 | 50, 400 | 11% | 11% | 6% | 5% | 4% | 3% |
| oxycodone 1 | IS oxycodone d3 | 50, 400 | 8% | 5% | 6% | 6% | 6% | 6% |
| oxymorphone 1 | IS oxymorphone d3 | 5, 40 | 9% | 6% | 8% | 6% | 6% | 5% |
| ramelteon 1 | IS alprazolam d5 | 5, 40 | 10% | 7% | 10% | 4% | 7% | 9% |
| temazepam 1 | IS temazepam d5 | 50, 400 | 9% | 10% | 6% | 4% | 7% | 6% |
| zaleplon 1 | IS zaleplon d4 | 50, 400 | 7% | 5% | 6% | 4% | 4% | 4% |
| zolpidem 1 | IS zolpidem d6 | 50, 400 | 2% | 3% | 2% | 2% | 2% | 1% |
| zopiclone 1 | IS zopiclone d4 | 50, 400 | 5% | 3% | 5% | 4% | 4% | 4% |

*Conclusion*

Several compounds demonstrated average suppression or enhancement greater than ± 25% for either the analyte, internal standard or both. None of the compounds demonstrated average suppression or enhancement greater than ± 11% for the relative response indicating that the use of isotopically-labeled internal standards compensated for any significant ion suppression or enhancement. Likewise the % CV demonstrated for the analyte and internal standard for methadone was greater than 15%, but the % CV for the relative response was not greater than 10% for any compound. The use of isotopically-labeled internal standards compensated for any significant ion suppression or enhancement.

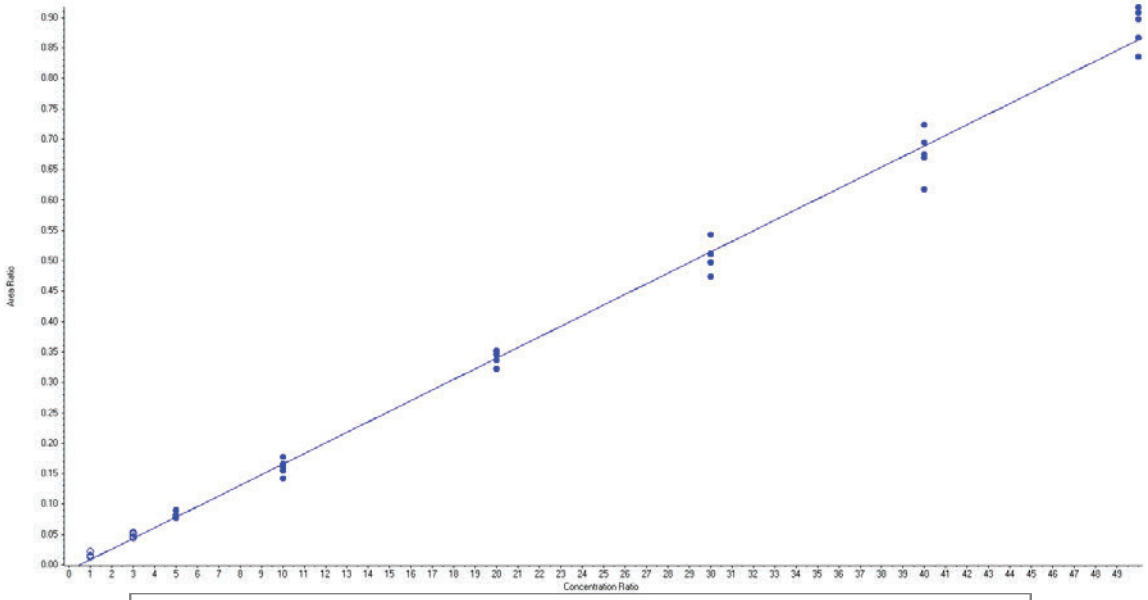
**Calibration model/linearity**

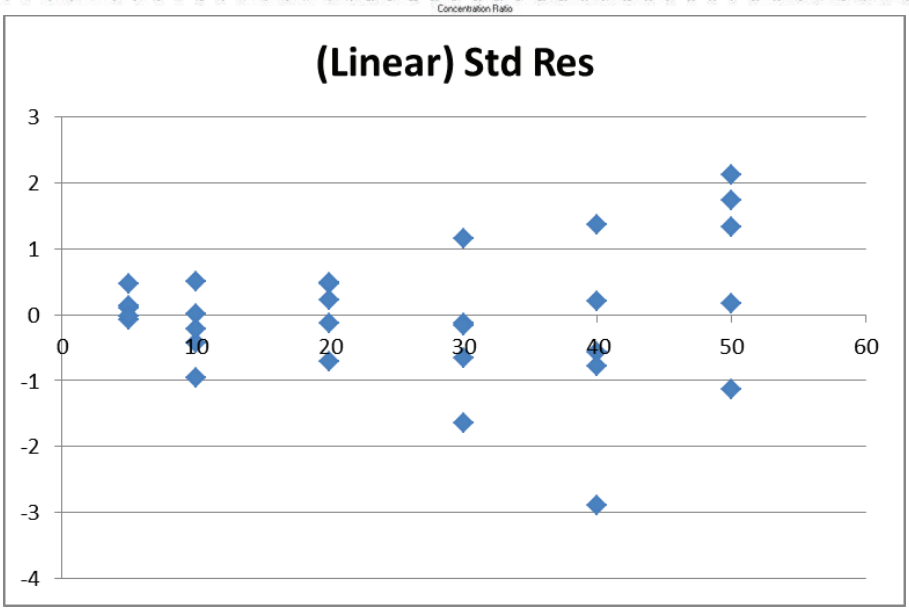
Calibrators at 11 concentrations from 0.1 to 100 ng/mL or 1 to 1000 ng/mL were prepared and extracted. The combined data of at least 6 non-zero concentrations evenly spaced across the calibration range with five replicates at each level analyzed in 5 separate extractions (one replicate per level per extraction) were used to establish the calibration model. The coefficient of determination ($r^2$) was ≥ 0.990 for all compounds except chlordiazepoxide. A more appropriate minimum coefficient of determination for chlordiazepoxide will be specified in the method as ≥ 0.980. Visual inspection of the curves and residual

plots indicated normal random scatter around the calibration curve. All calibrators were within ± 20% of their prepared concentration. Standardized residual plots for each compound validated for quantitative analysis were constructed. Any outliers (outside ± 3 standard deviations) were eliminated prior to final analysis of the standardized residual plots. Std Res was used as an abbreviation for standardized residual plot.

### 6-mam

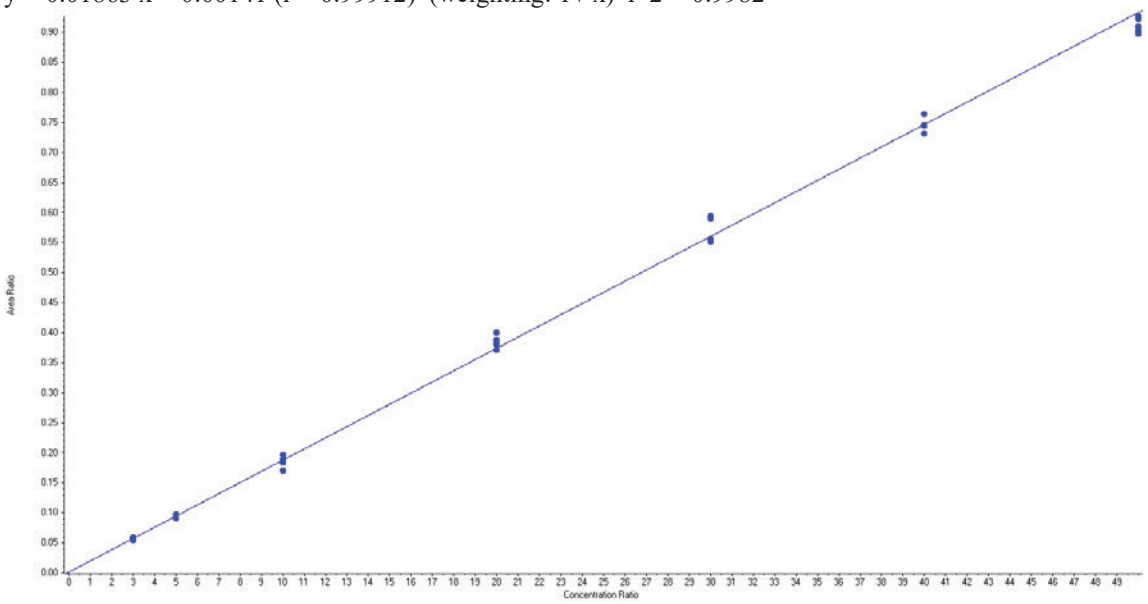
y = 0.01744 x + -0.00885 (r = 0.99746) (weighting: 1 / x) r^2 = 0.9949

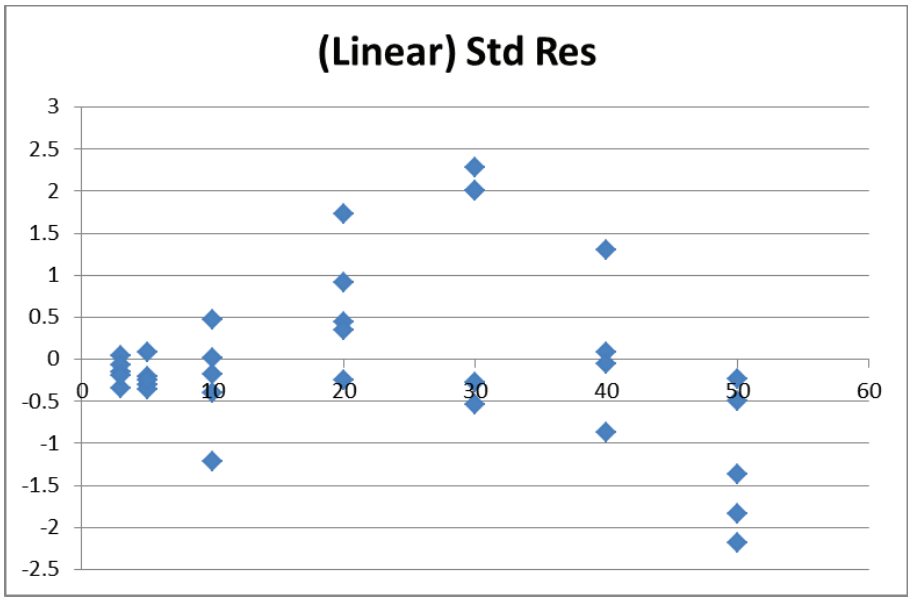


## 7-aminoflunitrazepam

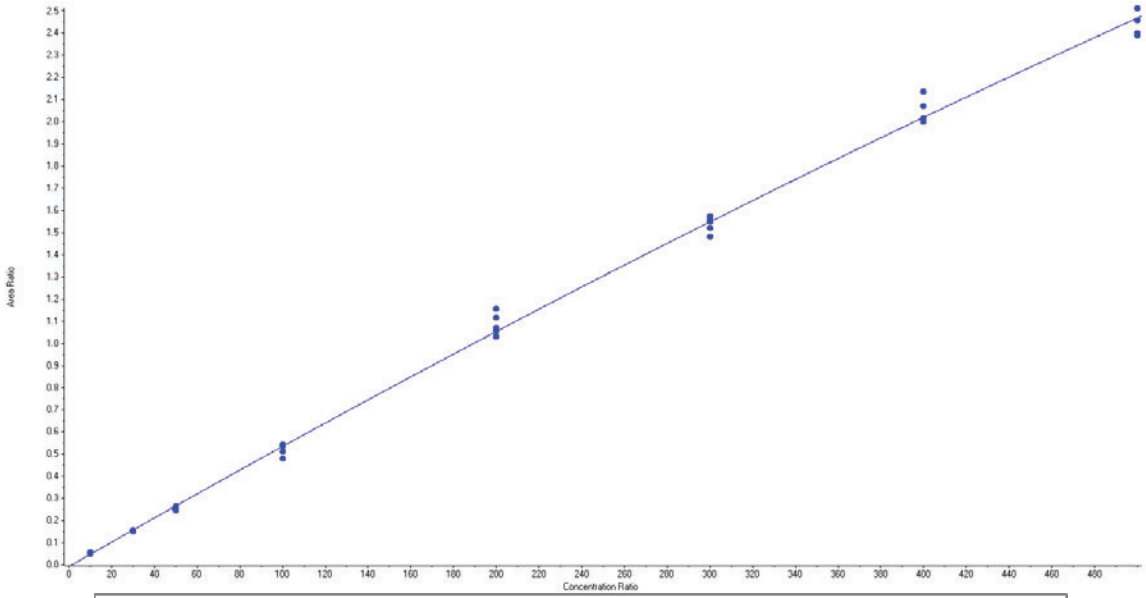
y = 0.01863 x + 0.00141 (r = 0.99912)  (weighting: 1 / x)  r^2 = 0.9982

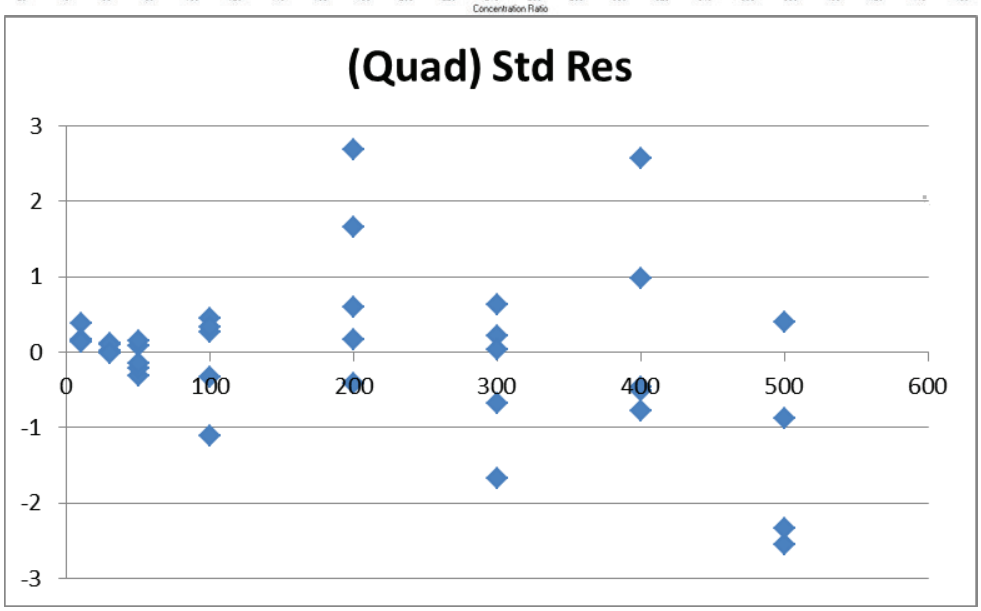


## Alprazolam

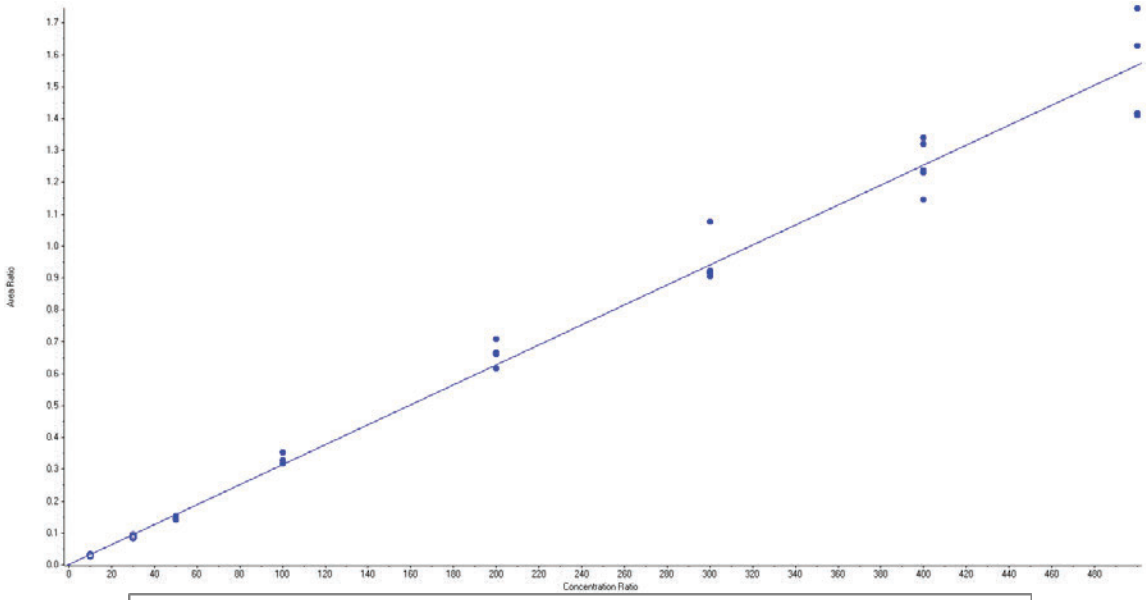
y = -1.18125e-6 x^2 + 0.00554 x + -0.00754 (r = 0.99923)  (weighting: 1 / x)  r^2 = 0.9985

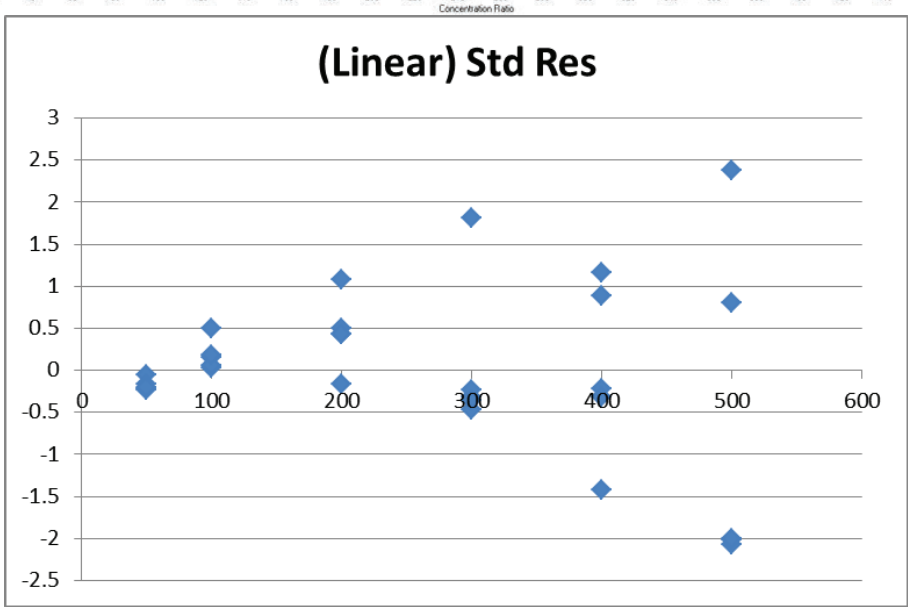


## Chlordiazepoxide

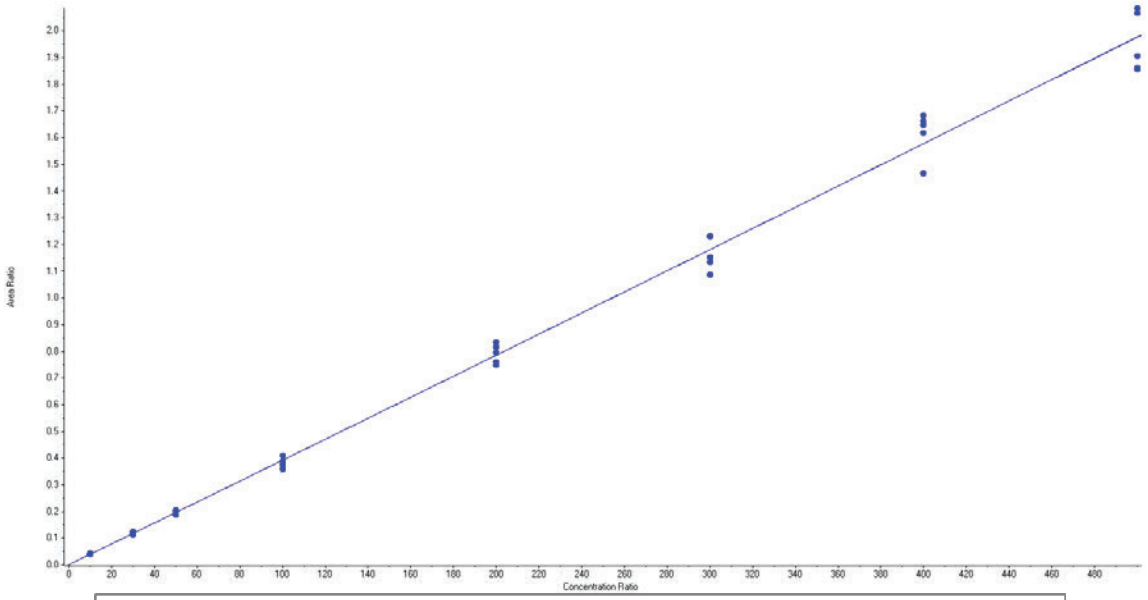
y = 0.00313 x + 0.00127 (r = 0.99385)  (weighting: 1 / x)  r^2 = 0.9877

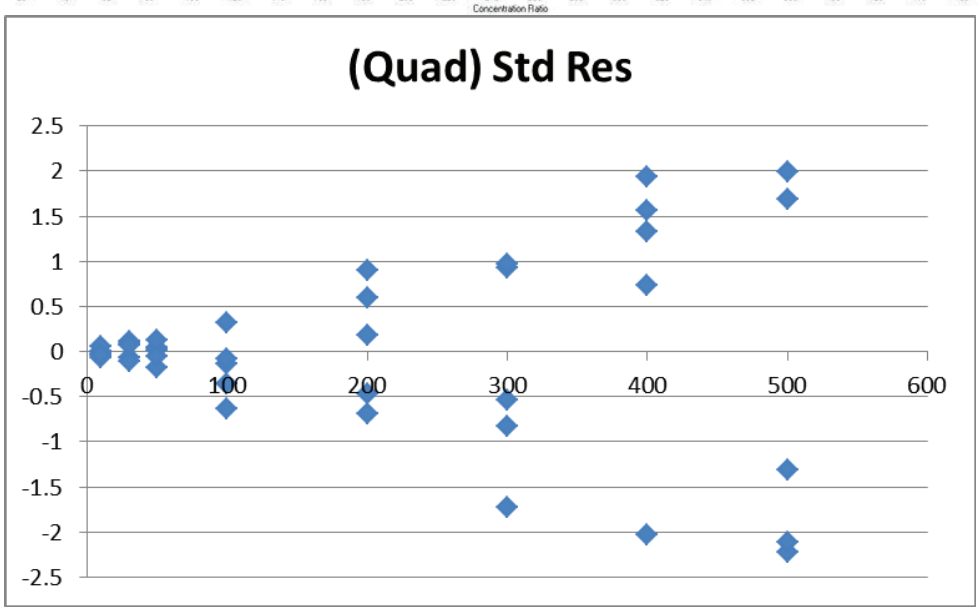


## Clonazepam

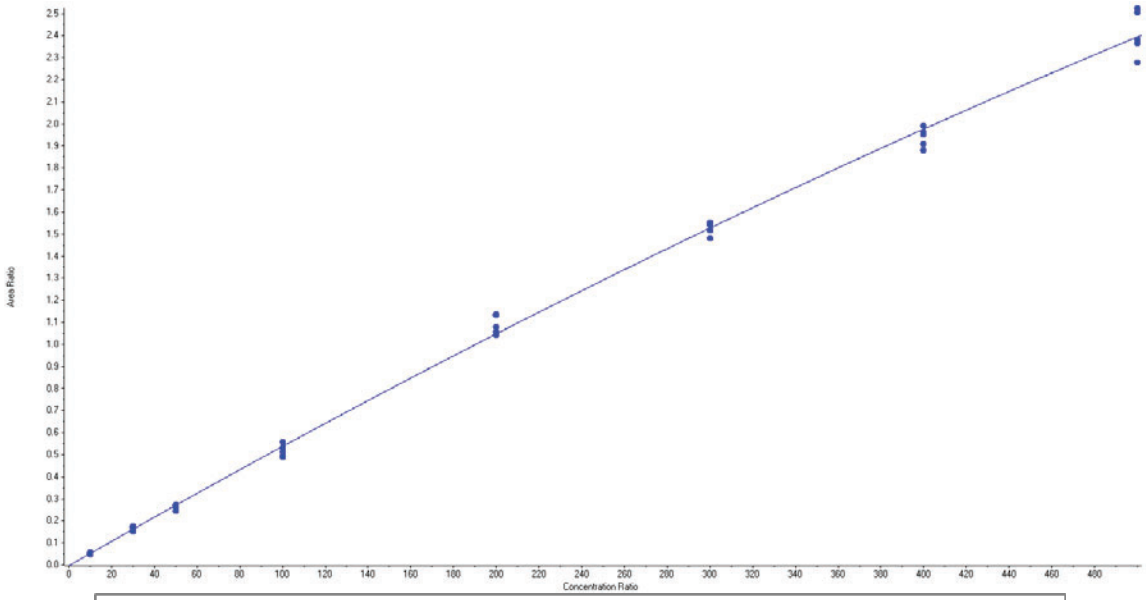
y = 9.73182e-8 x^2 + 0.00390 x + 8.72523e-4 (r = 0.99838)  (weighting: 1 / x)  r^2 = 0.9968



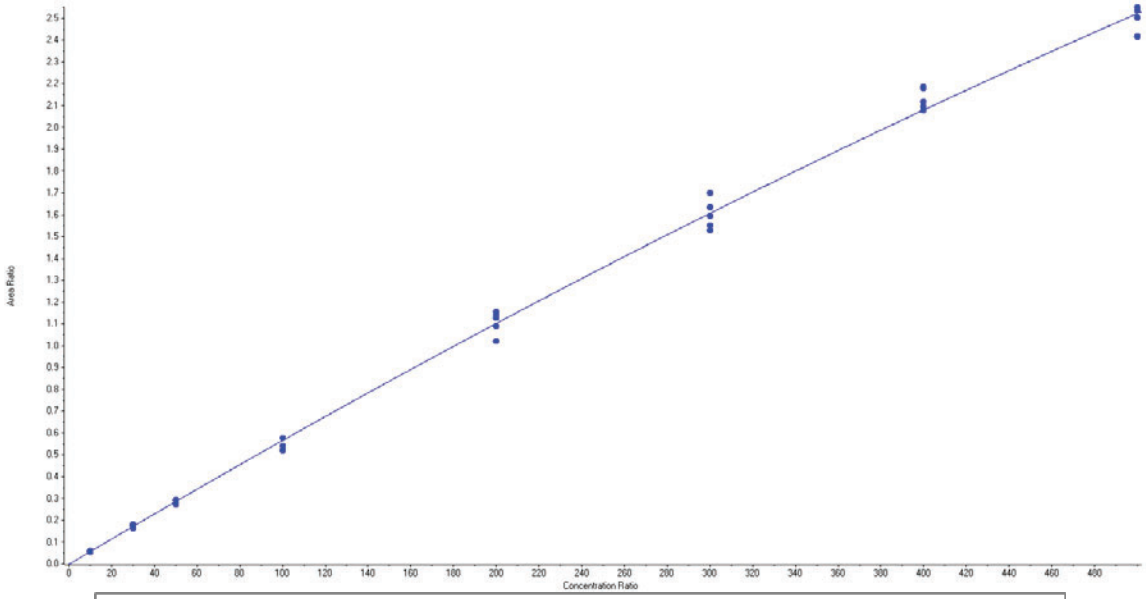
## Codeine

y = -1.53711e-6 x^2 + 0.00557 x + -0.00364 (r = 0.99900)  (weighting: 1 / x)  r^2 = 0.9980



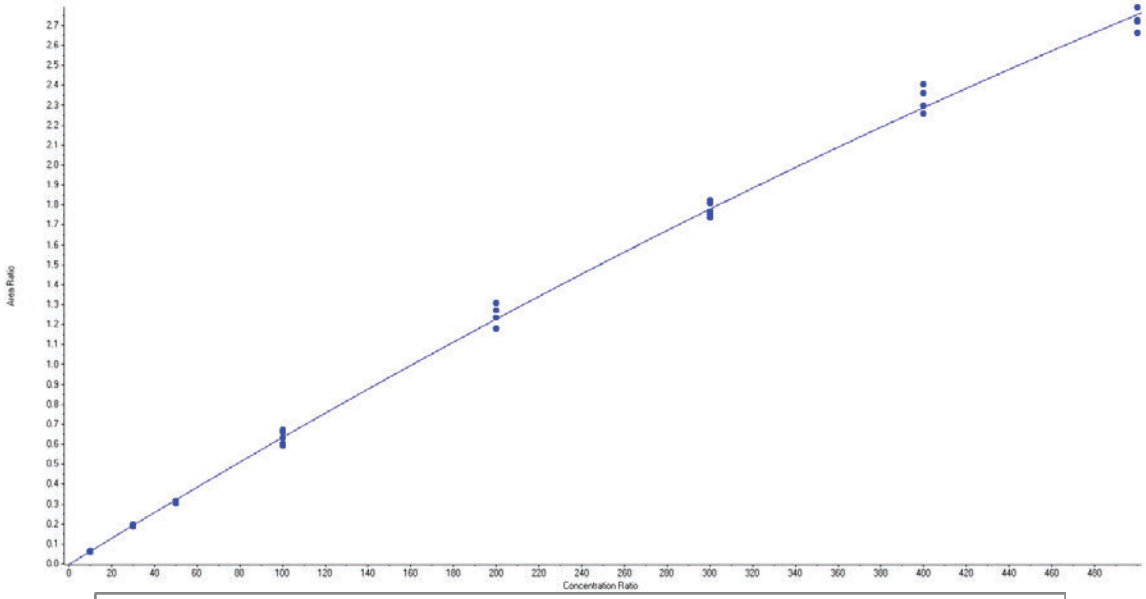

## Desalkylflurazepam

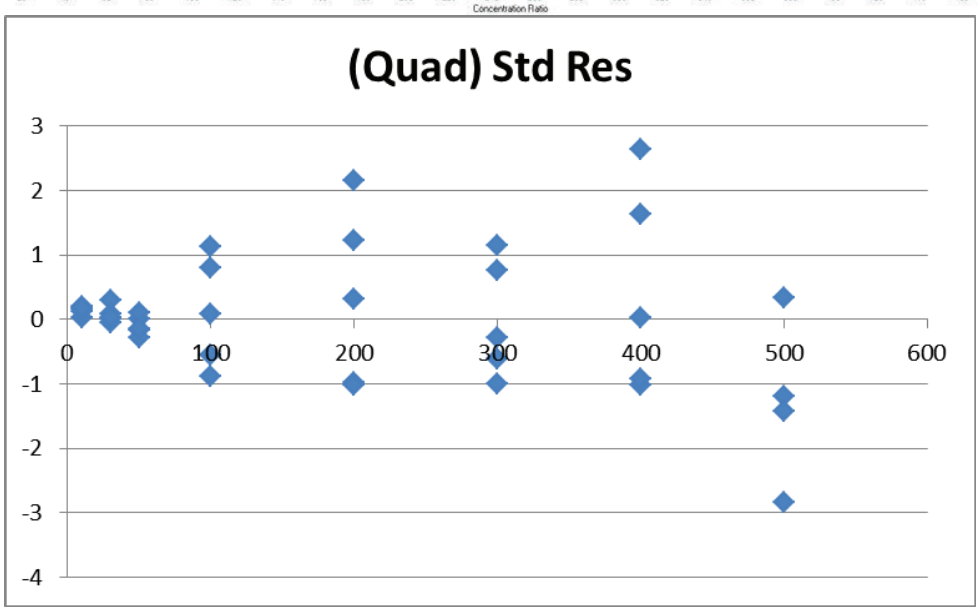
y = -1.56839e-6 x^2 + 0.00583 x + -0.00178 (r = 0.99911)  (weighting: 1 / x)  r^2 = 0.9982




## Diazepam

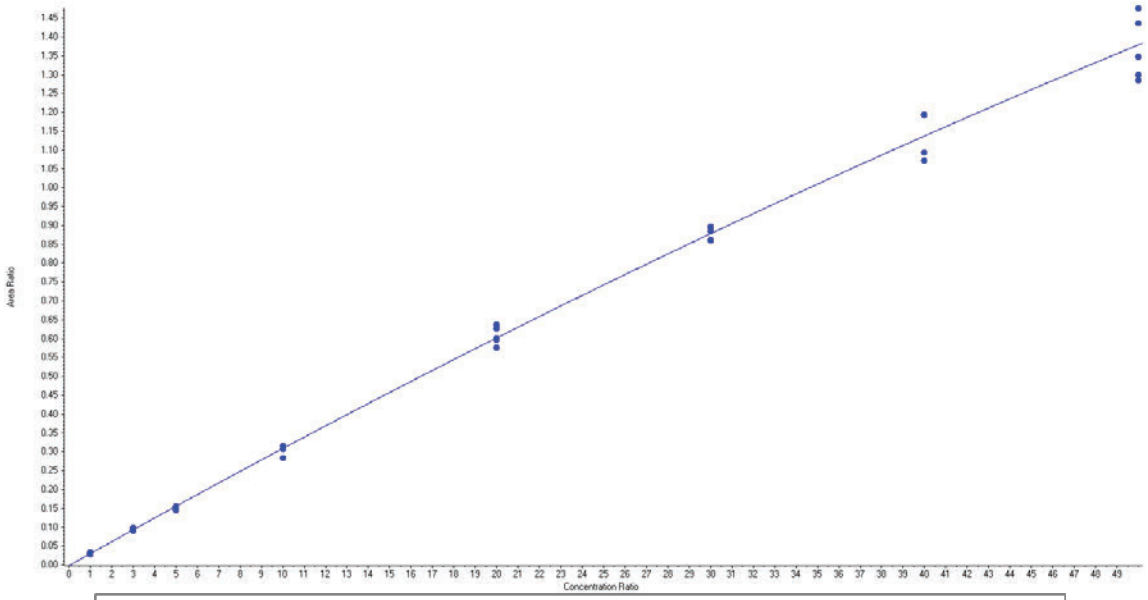
y = -2.12794e-6 x^2 + 0.00658 x + -0.00255 (r = 0.99941)  (weighting: 1 / x)  r^2 = 0.9988

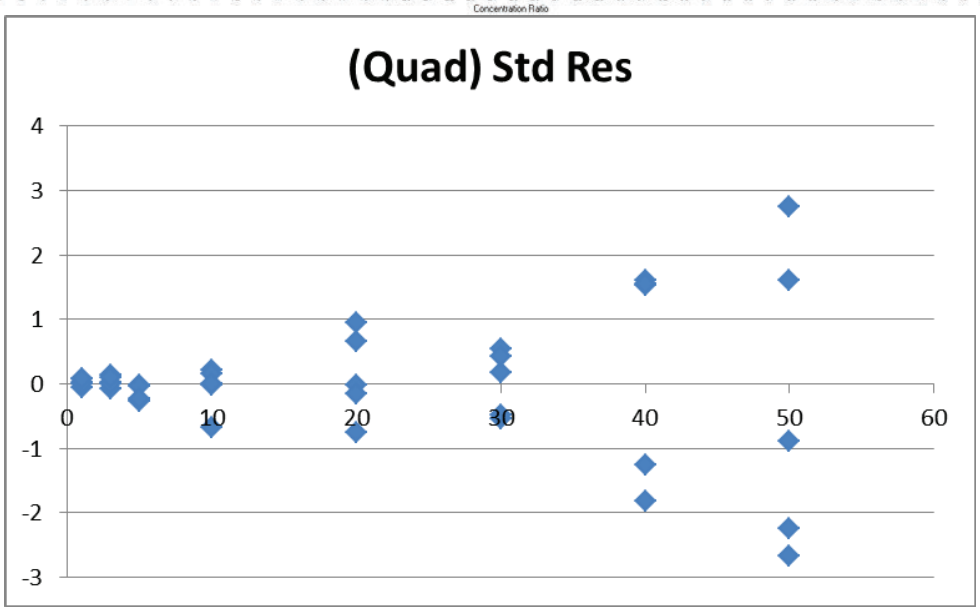


## Fentanyl

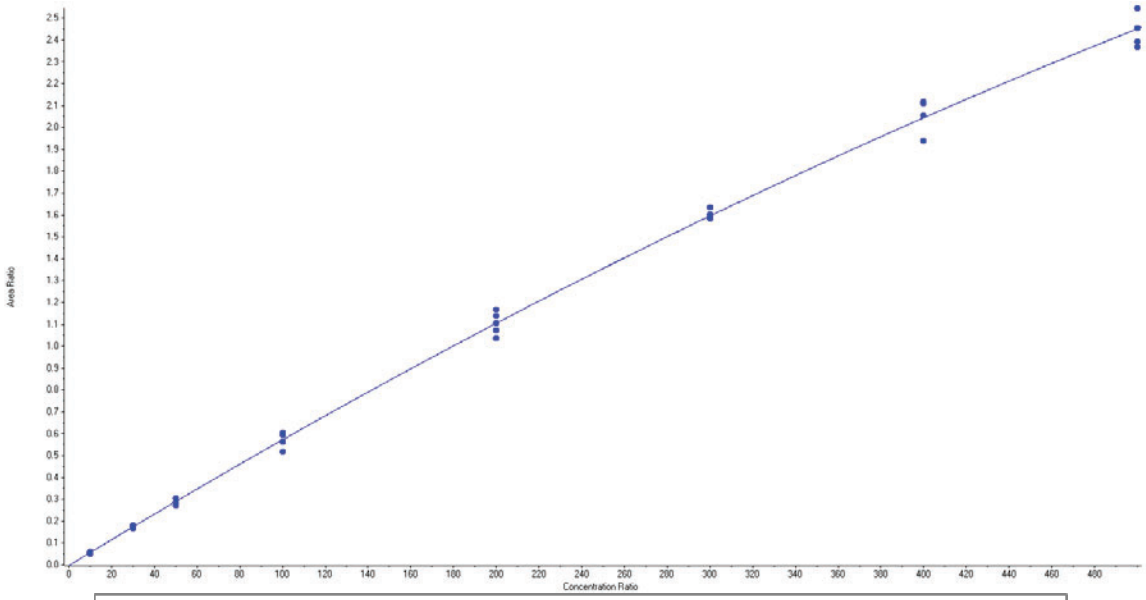
y = -8.62532e-5 x^2 + 0.03193 x + -0.00194 (r = 0.99873)  (weighting: 1 / x)  r^2 = 0.9975

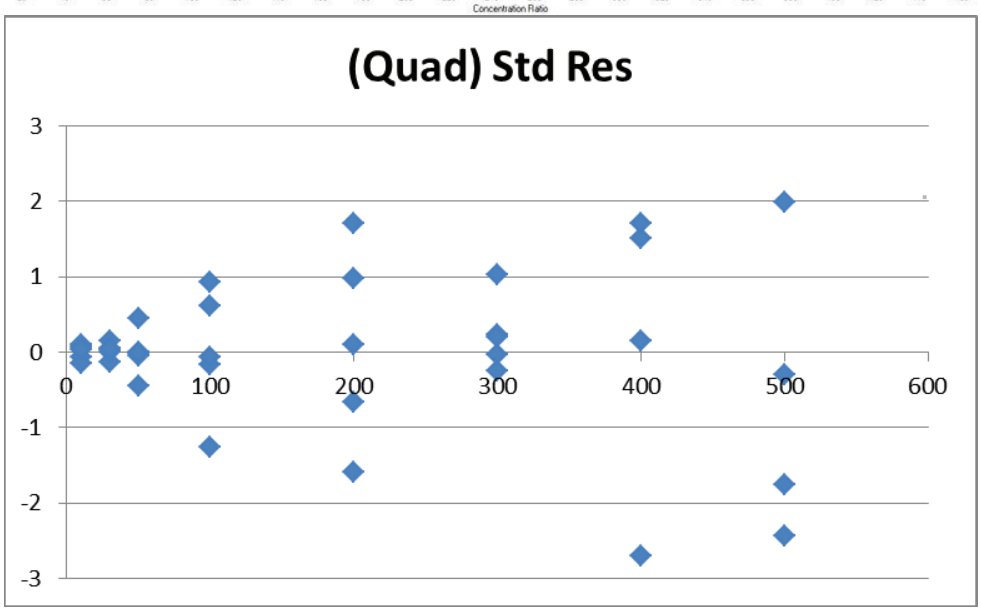


## Hydrocodone

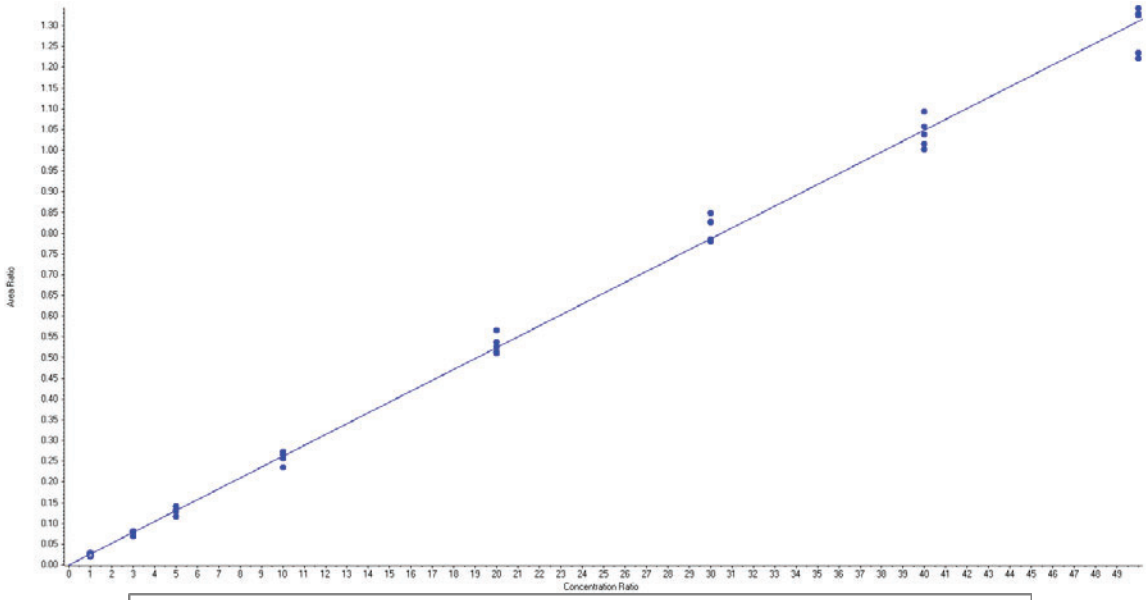
y = -2.10319e-6 x^2 + 0.00596 x + -0.00278 (r = 0.99924)  (weighting: 1 / x)  r^2 = 0.9985

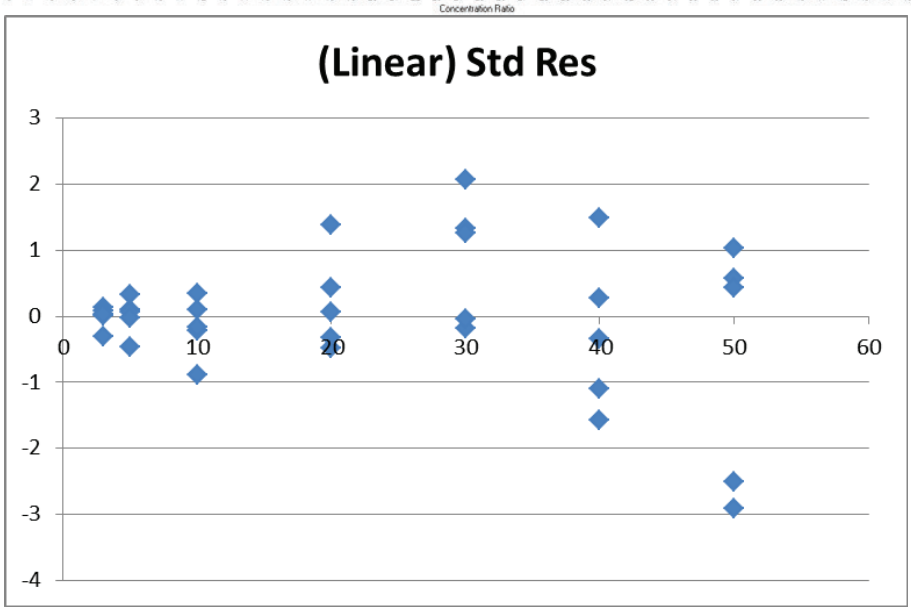


## Hydromorphone

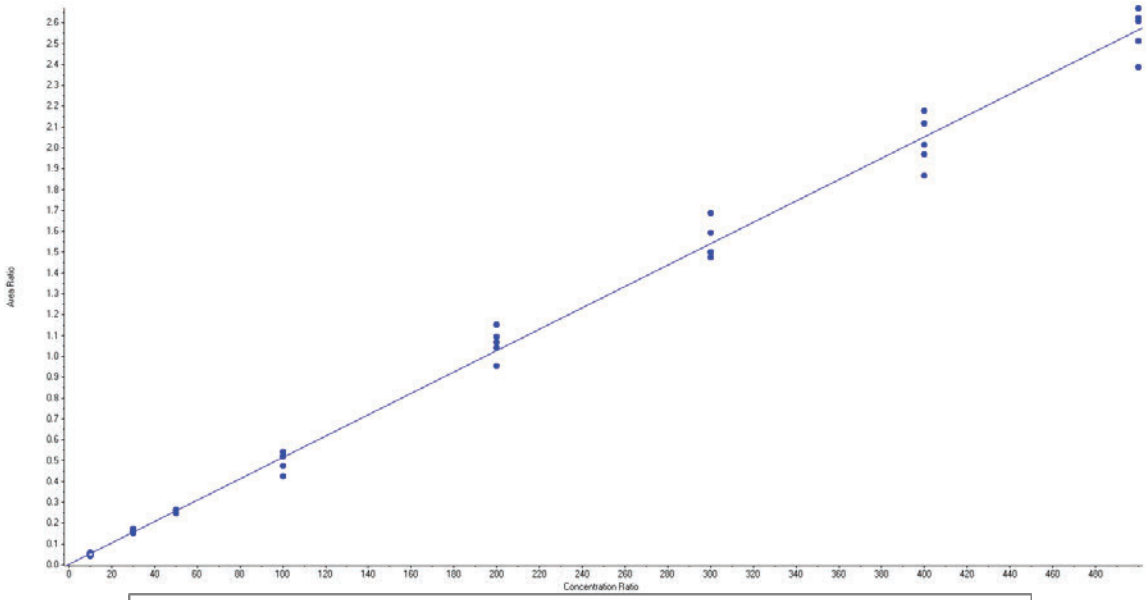
y = 0.02622 x + -1.24577e-4 (r = 0.99848)  (weighting: 1 / x)  r^2 = 0.9970

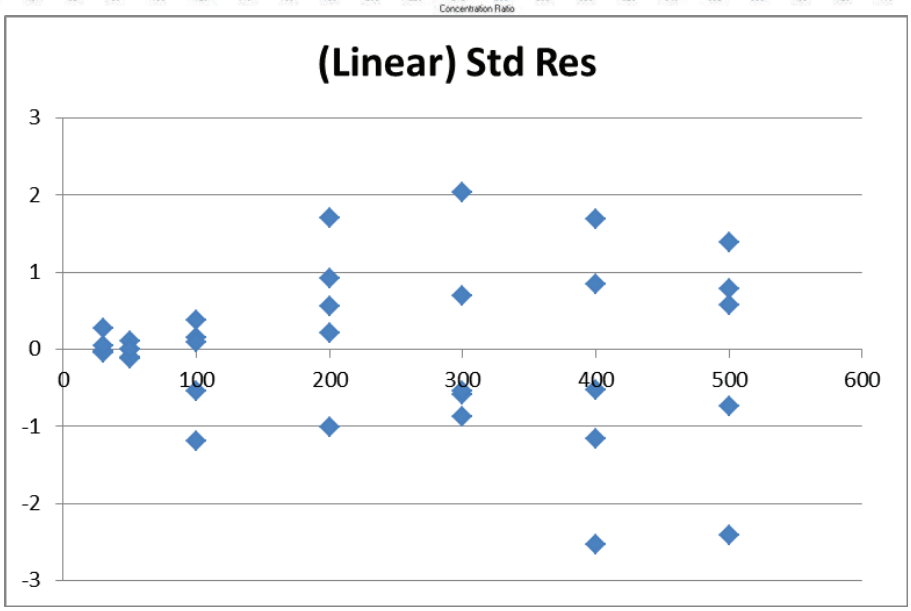


## Lorazepam

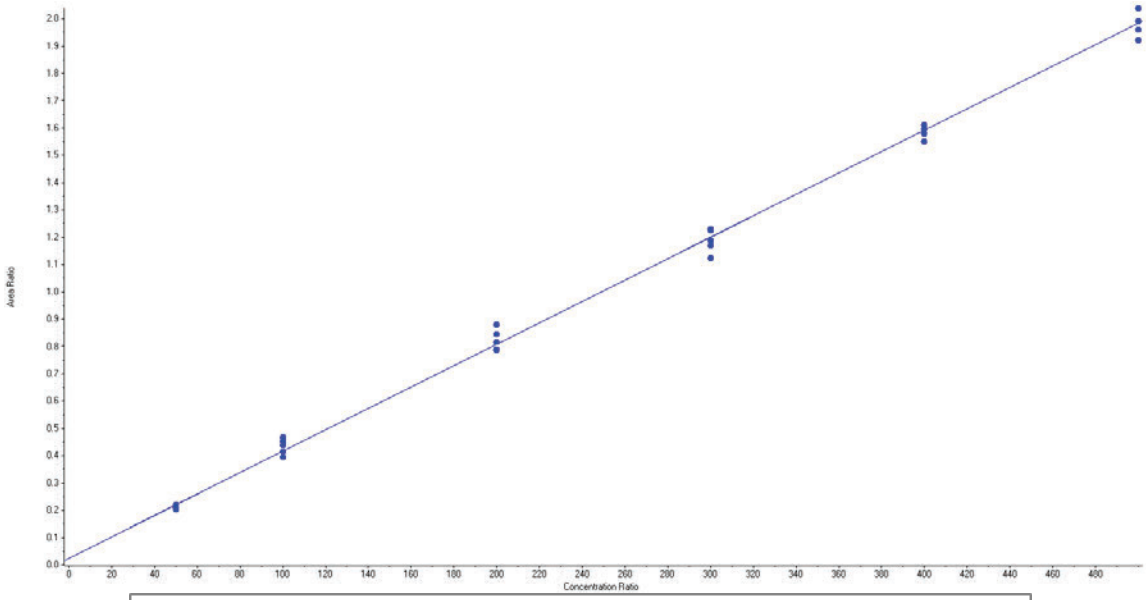
y = 0.00513 x + 0.00265 (r = 0.99748)  (weighting: 1 / x)  r^2 = 0.9950

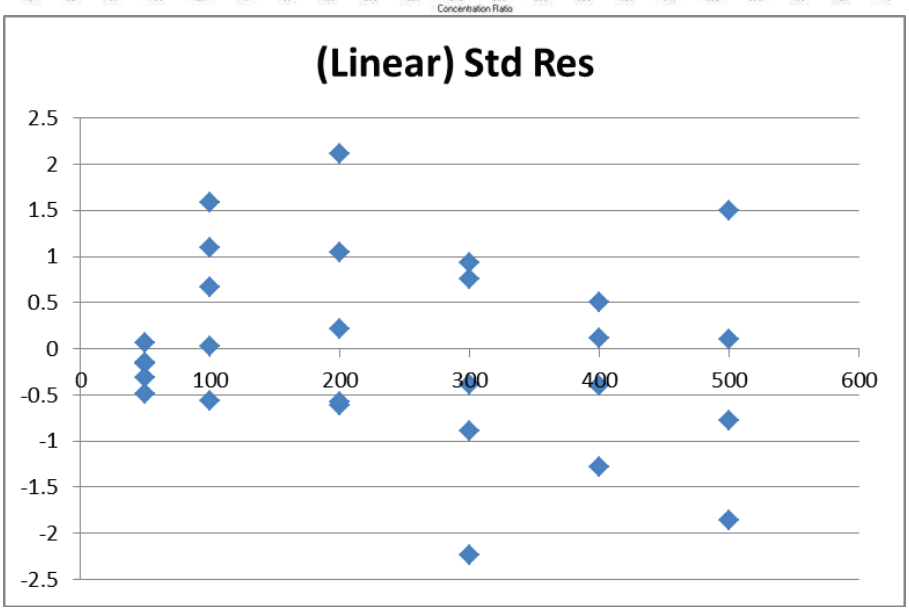


## Methadone

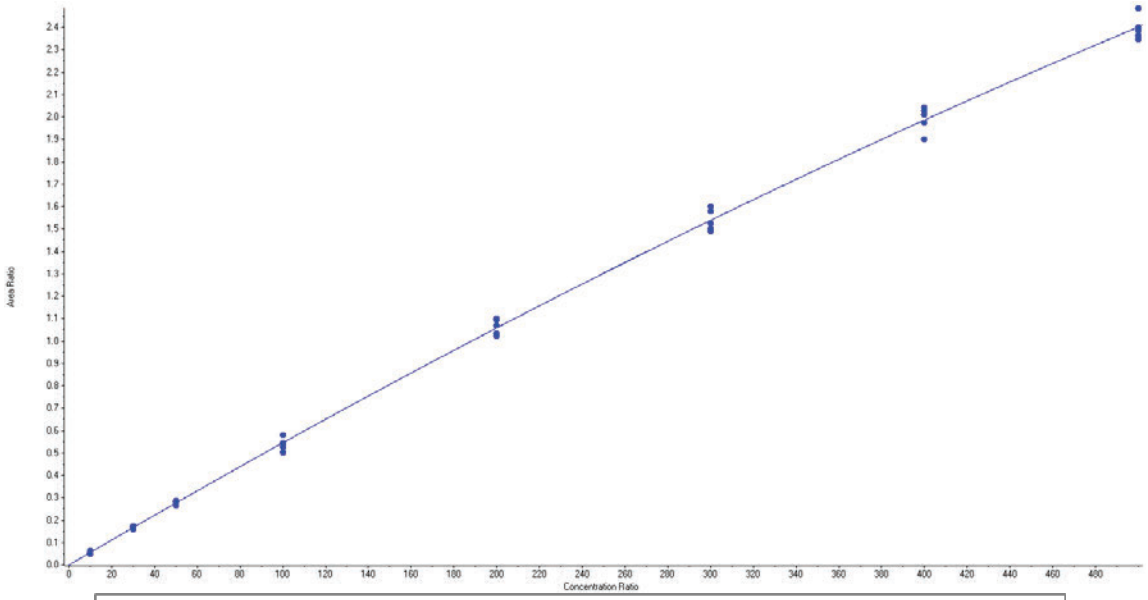
y = 0.00392 x + 0.02451 (r = 0.99847)  (weighting: 1 / x)  r^2 = 0.9969

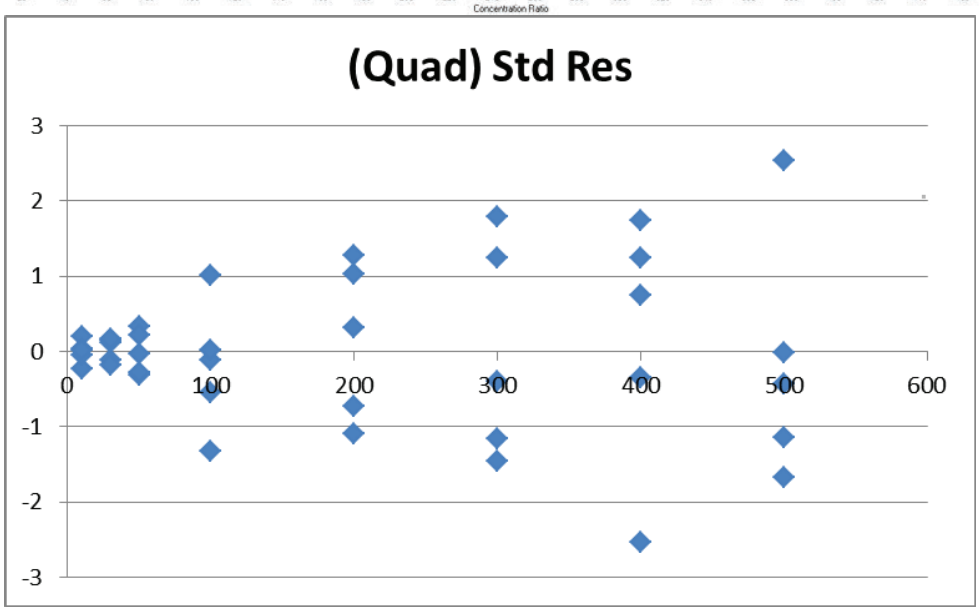


## Midazolam

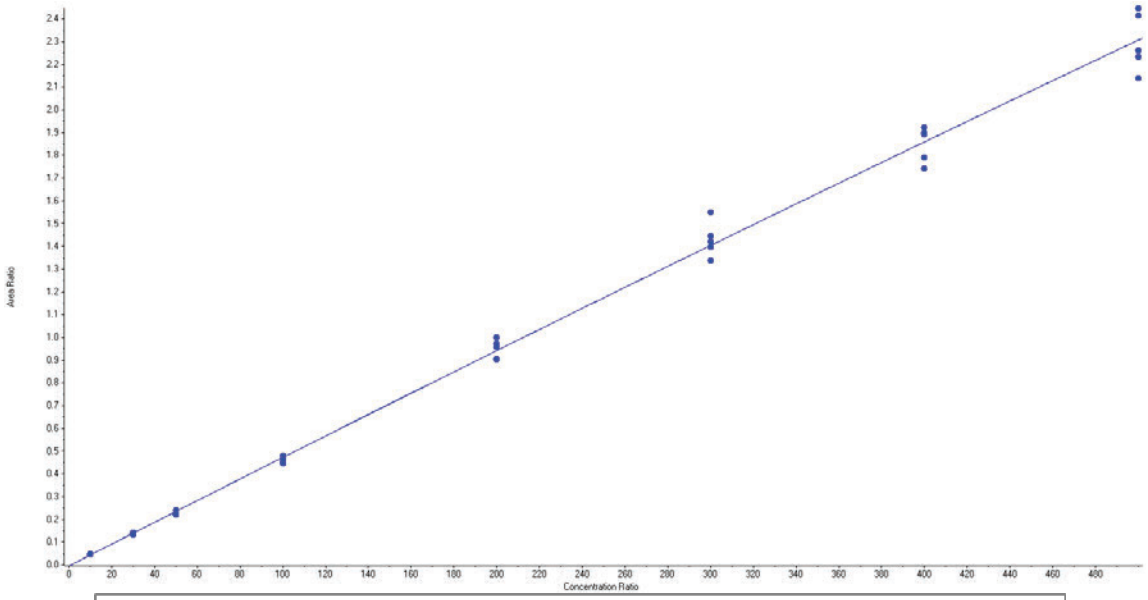
y = -1.63298e-6 x^2 + 0.00562 x + -3.59192e-4 (r = 0.99945)  (weighting: 1 / x)  r^2 = 0.9989



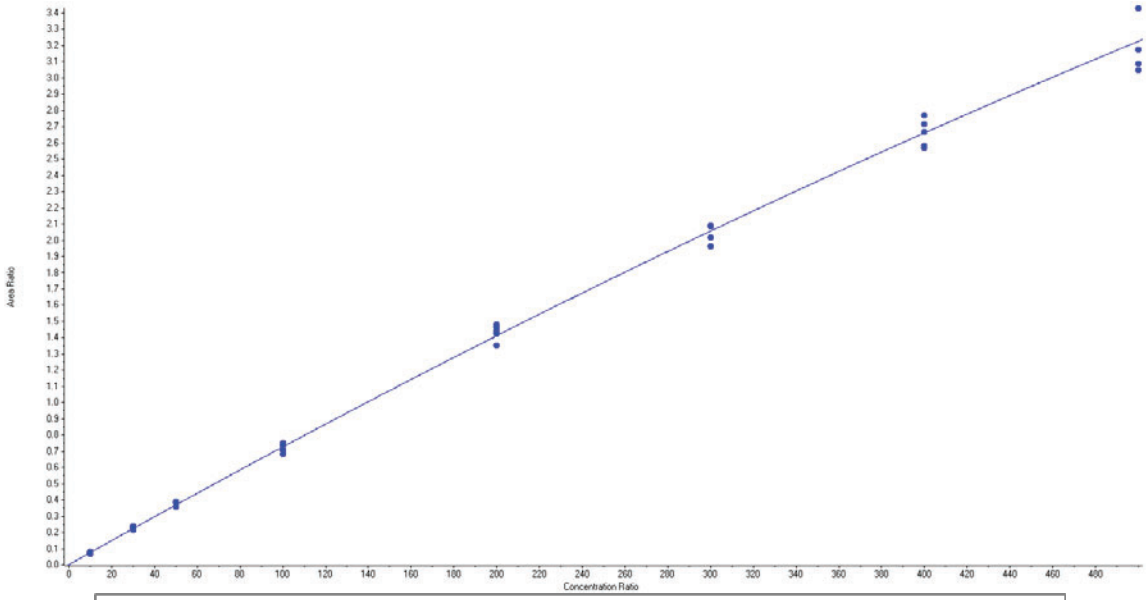
## Morphine

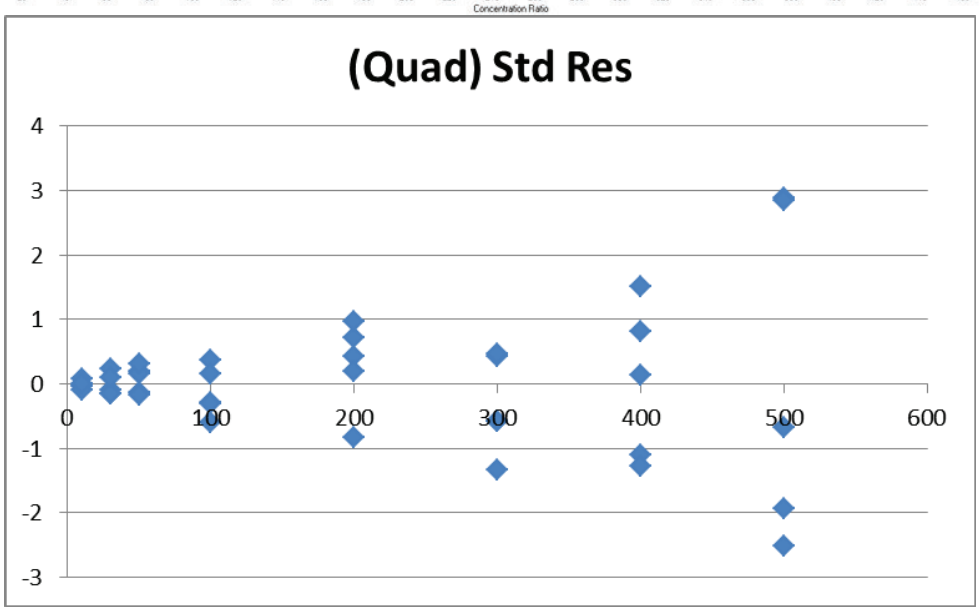
y = -3.62112e-7 x^2 + 0.00480 x + -0.00419 (r = 0.99868)  (weighting: 1 / x)  r^2 = 0.9974




## Nordiazepam

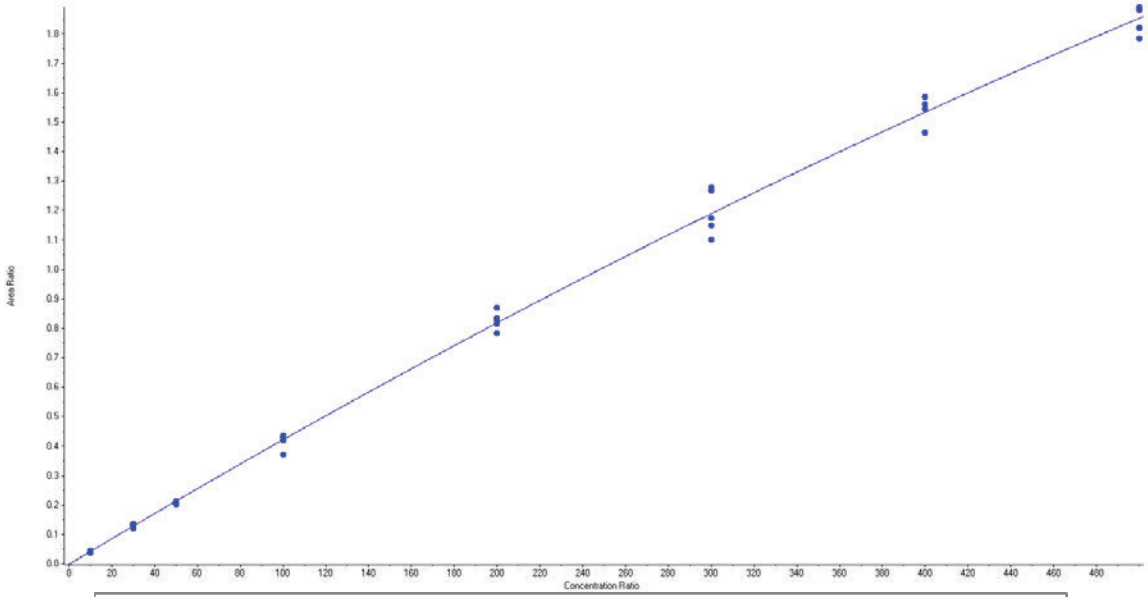
y = -2.01165e-6 x^2 + 0.00745 x + 0.00198 (r = 0.99903)  (weighting: 1 / x)  r^2 = 0.9981

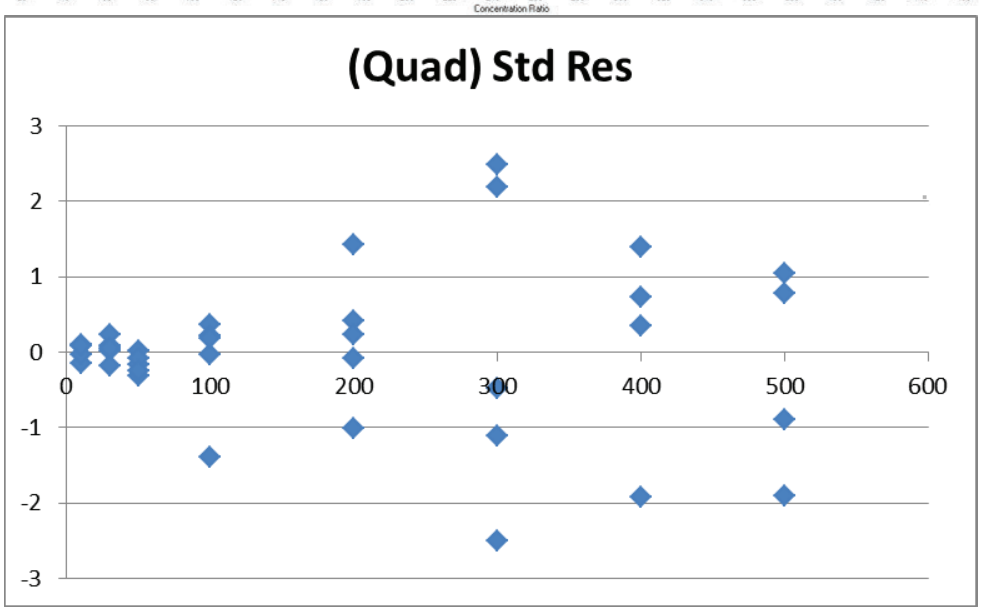


## Oxazepam

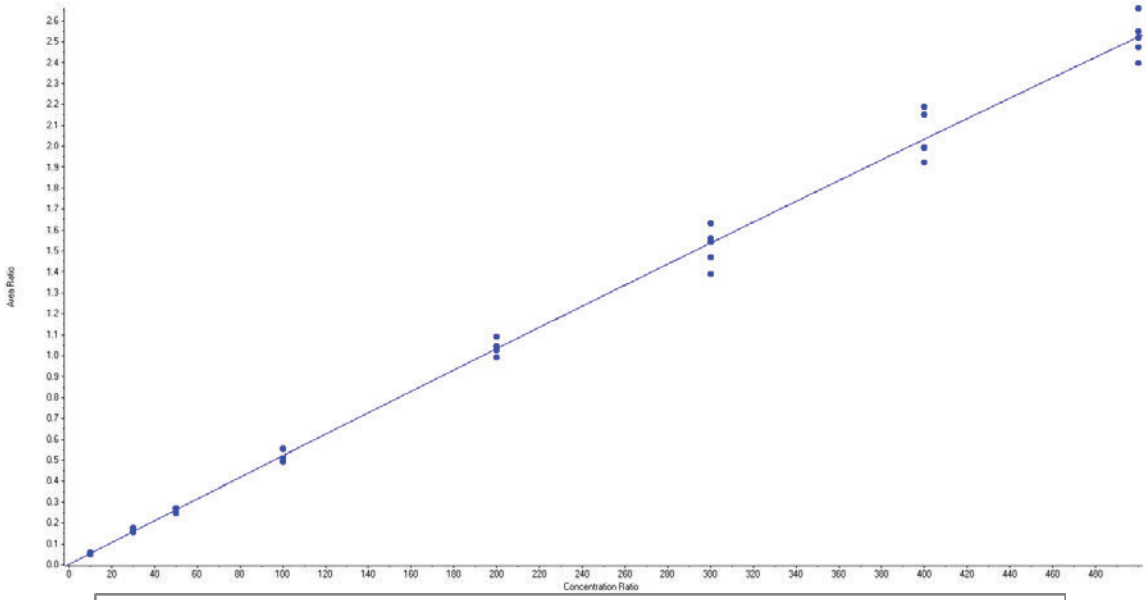
y = -1.30223e-6 x^2 + 0.00436 x + -0.00120 (r = 0.99889)  (weighting: 1 / x)  r^2 = 0.9978

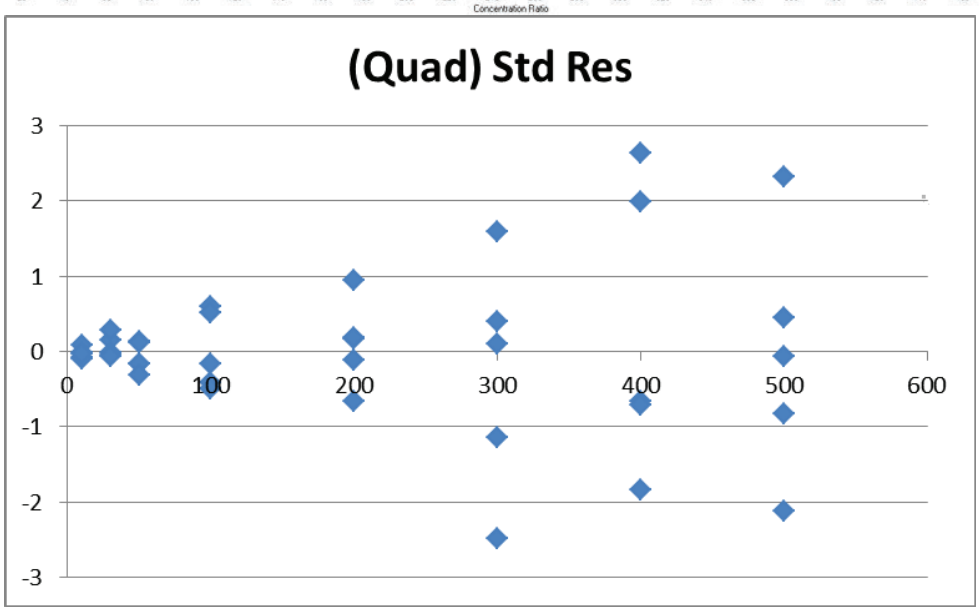


## Oxycodone

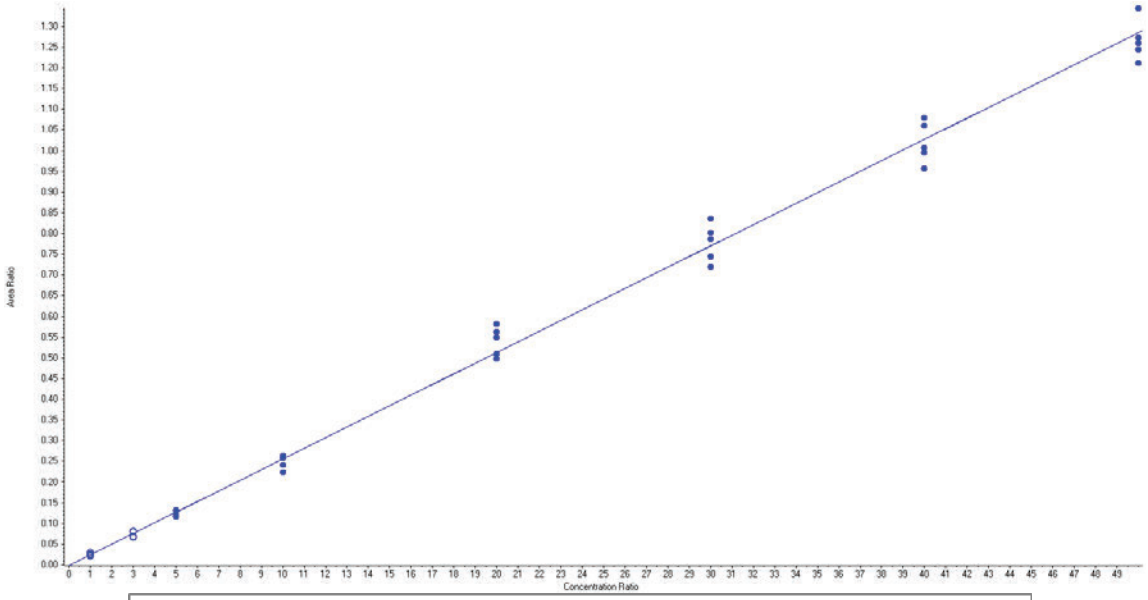
y = -3.85084e-7 x^2 + 0.00523 x + 0.00234 (r = 0.99867)  (weighting: 1 / x)  r^2 = 0.9973

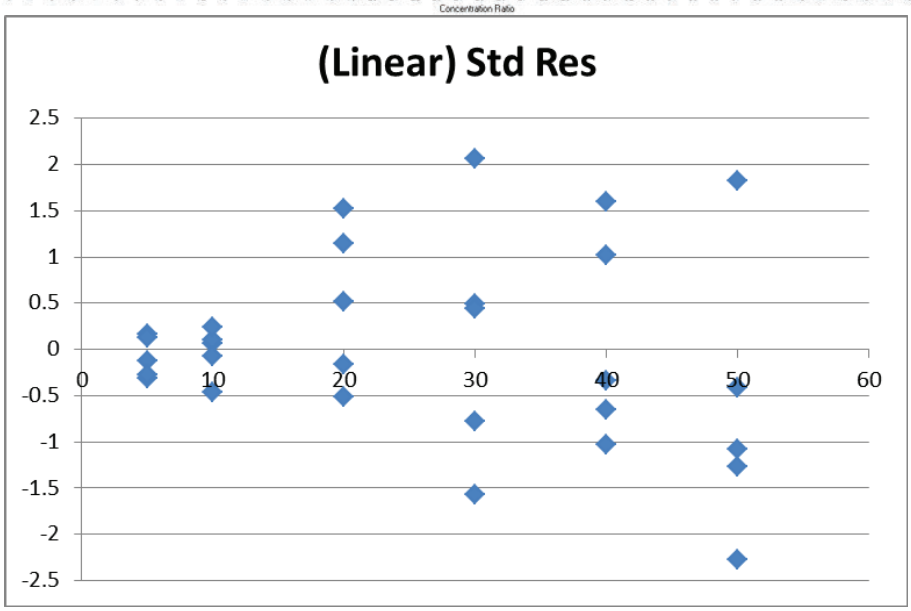


## Oxymorphone

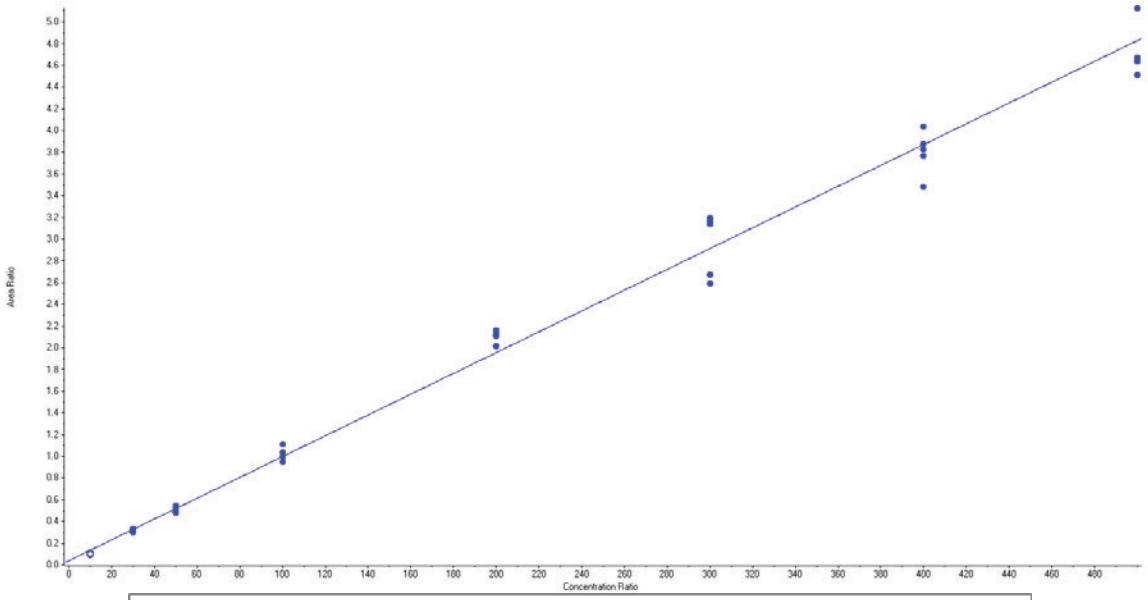
y = 0.02572 x + -0.00142 (r = 0.99707)  (weighting: 1 / x)  r^2 = 0.9941

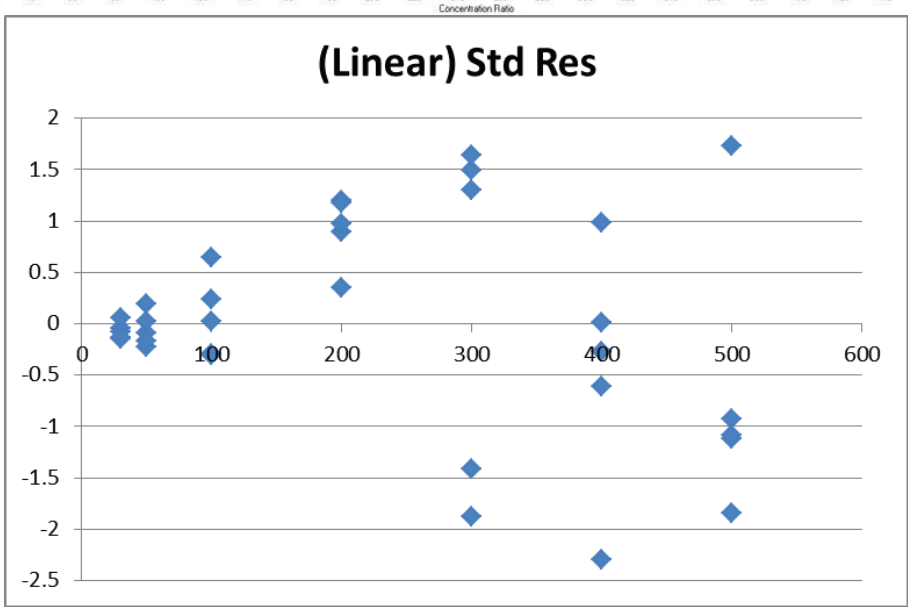


## Temazepam

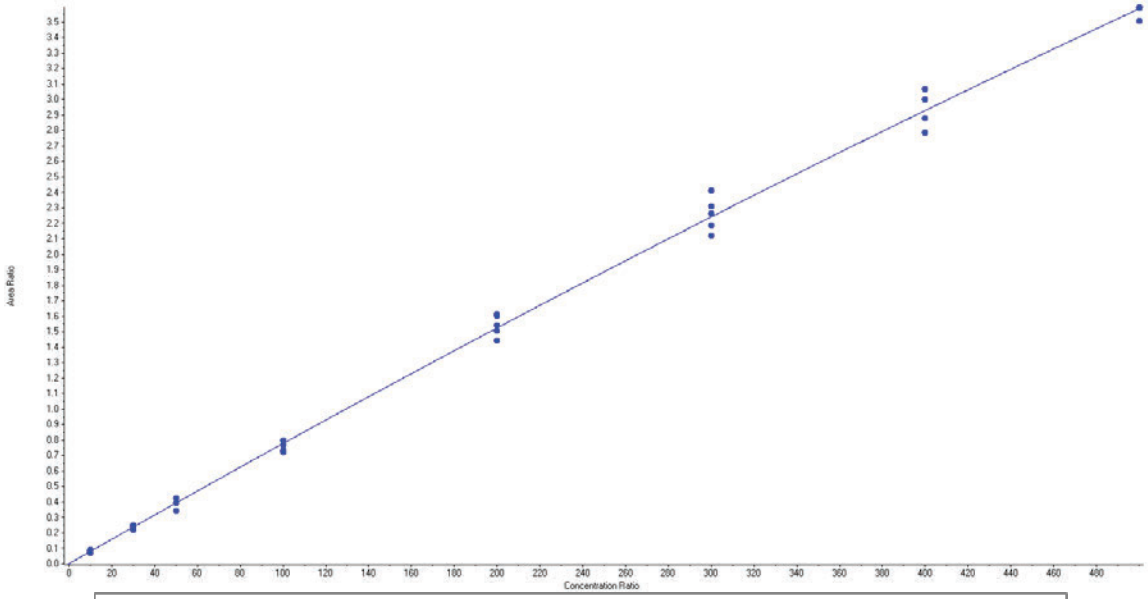
y = 0.00958 x + 0.03998 (r = 0.99625)  (weighting: 1 / x)  r^2 = 0.9925



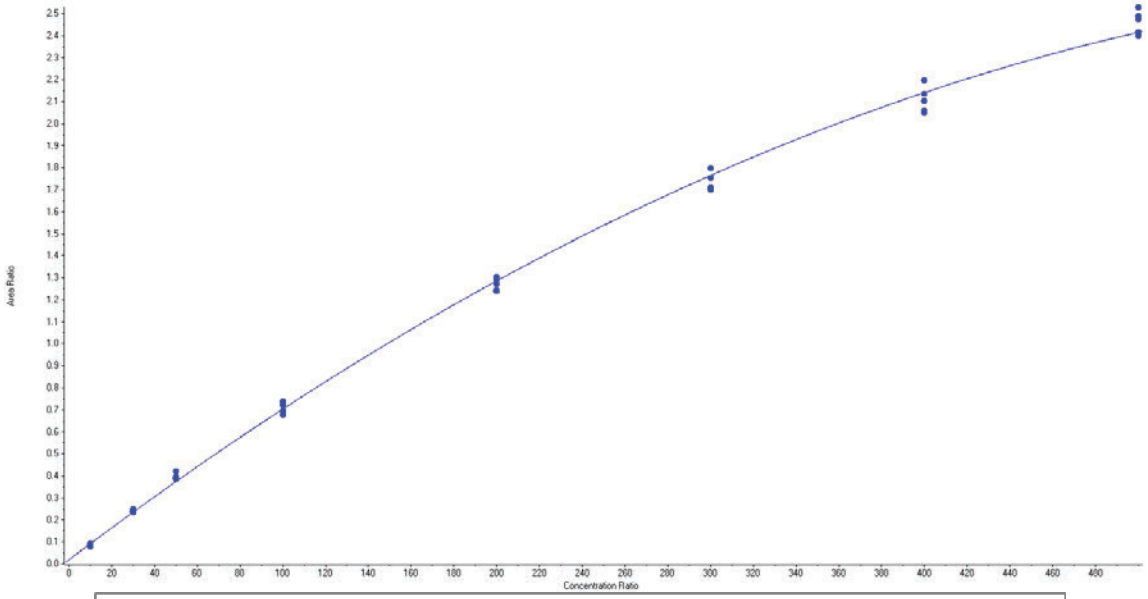
## Zaleplon

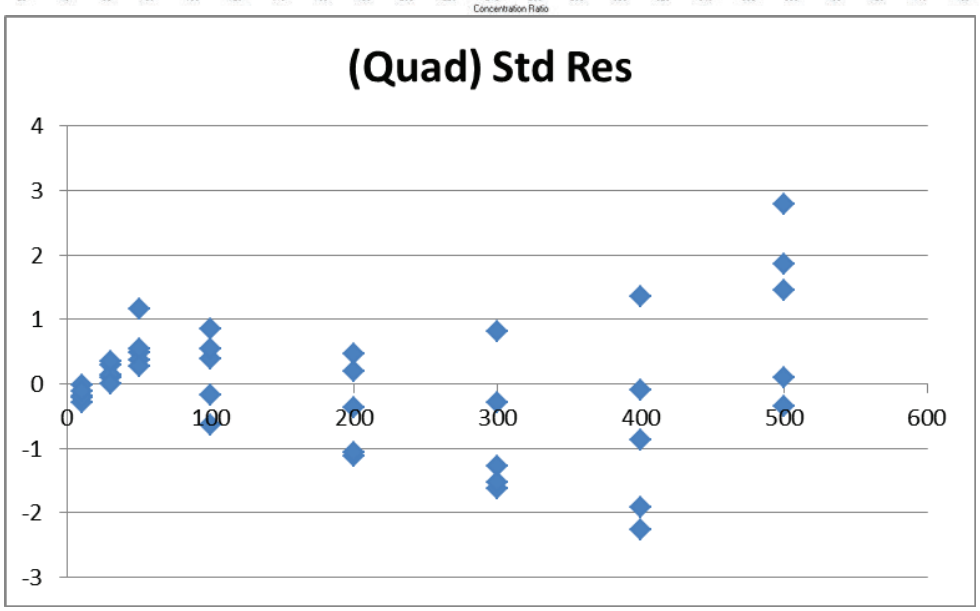
y = -1.48248e-6 x^2 + 0.00791 x + 0.00201 (r = 0.99891)  (weighting: 1 / x)  r^2 = 0.9978




## Zolpidem

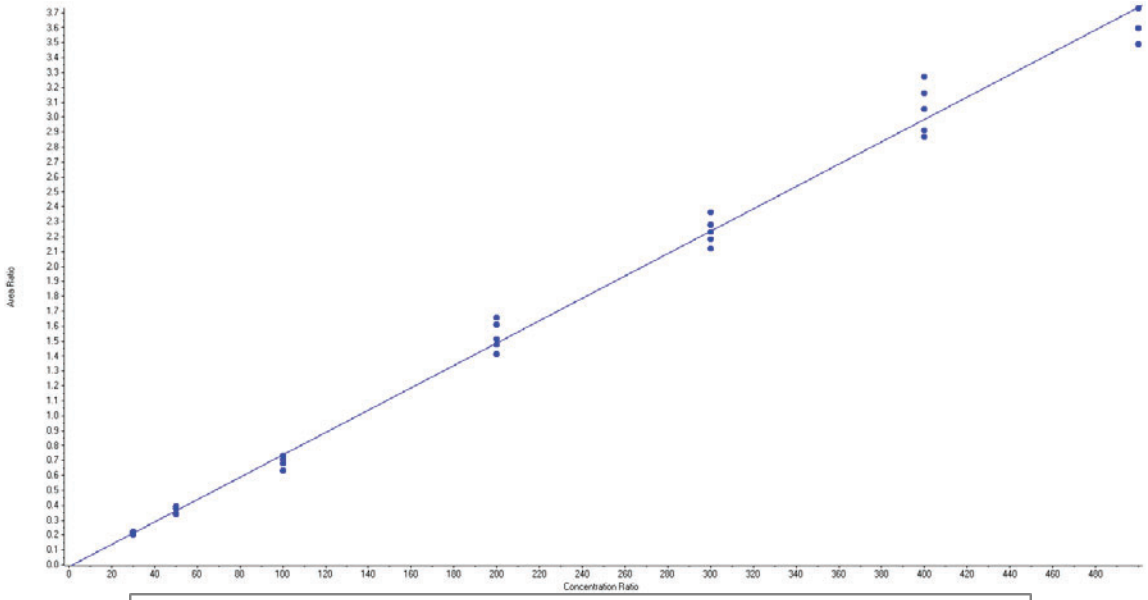
y = -5.14154e-6 x^2 + 0.00736 x + 0.01873 (r = 0.99916)  (weighting: 1 / x)  r^2 = 0.9983

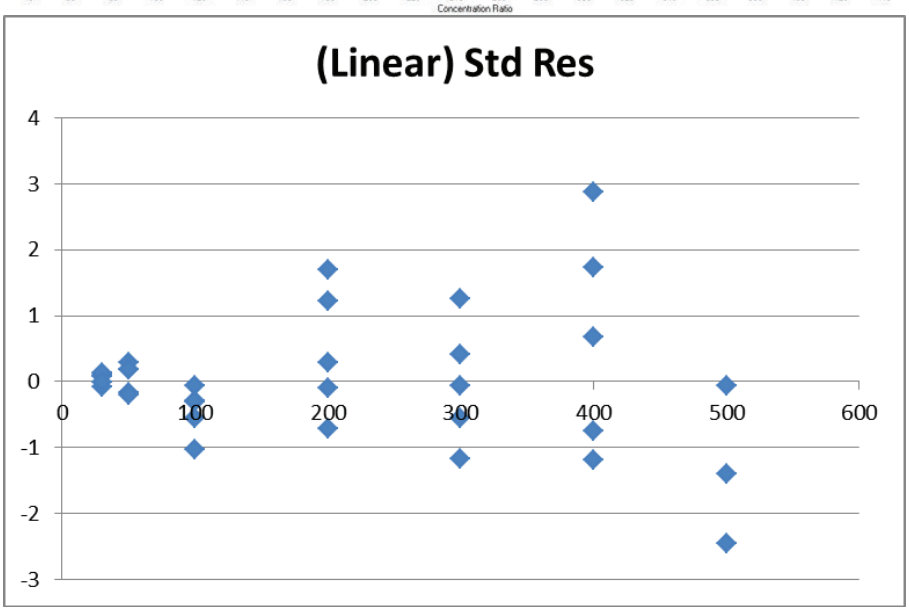


## Zopiclone

y = 0.00750 x + -0.01219 (r = 0.99762)  (weighting: 1 / x)  r^2 = 0.9952




*Conclusion*

A suitable calibration model was determined for each compound that was validated for quantitative analysis and is summarized below.

| Analyte | Range(ng/mL) | Curve Fit | Weighting |
|---|---|---|---|
| 6-MAM | 5-50 | Linear | 1/x |
| 7-Aminoflunitrazepam | 3-50 | Linear | 1/x |
| Alprazolam | 10-500 | Quadratic | 1/x |
| Chlordiazepoxide | 50-500 | Linear | 1/x |
| Clonazepam | 10-500 | Quadratic | 1/x |
| Codeine | 10-500 | Quadratic | 1/x |
| Desalkylflurazepam | 10-500 | Quadratic | 1/x |
| Diazepam | 10-500 | Quadratic | 1/x |
| Dihydrocodeine | Qualitative Only | | |
| Fentanyl | 1-50 | Quadratic | 1/x |
| Flunitrazepam | Qualitative Only | | |
| Hydrocodone | 10-500 | Quadratic | 1/x |
| Hydromorphone | 3-50 | Linear | 1/x |
| Lorazepam | 30-500 | Linear | 1/x |
| Methadone | 50-500 | Linear | 1/x |
| Midazolam | 10-500 | Quadratic | 1/x |
| Morphine | 10-500 | Quadratic | 1/x |
| Norbuprenorphine | 3-50 | Linear | 1/x |
| Nordiazepam | 10-500 | Quadratic | 1/x |
| Oxazepam | 10-500 | Quadratic | 1/x |
| Oxycodone | 10-500 | Quadratic | 1/x |
| Oxymorphone | 5-50 | Linear | 1/x |
| Temazepam | 30-500 | Linear | 1/x |
| Zaleplon | 10-500 | Quadratic | 1/x |
| Zolpidem | 10-500 | Quadratic | 1/x |
| Zopiclone | 30-500 | Linear | 1/x |

**Sensitivity – Limit of Detection (LOD)**

The validated LOD was verified by at least two replicates of a standard run in five separate extractions prepared in whole blood containing a mixture of four different sources. All compounds had good chromatographic peak shape at the LOD. All signal-to-noise ratios were greater than 20:1. MRM ion ratios were within 20% and retention times were within 0.1 minutes compared to the average of all calibrators used (specific to each compound). An experimental LOD was determined by analyzing at least one replicate of a standard in five separate extractions prepared in whole blood containing a mixture of four different sources. Additional experiments may be performed to validate the determined experimental LOD.

| Analyte | Experimental LOD (ng/mL) | Lowest Signal to Noise at Experimental LOD | Validated LOD (ng/mL) | Lowest Signal to Noise at Validated LOD |
|---|---|---|---|---|
| 6-MAM | 5 | 205.6 | 5 | 205.6 |
| 7-Aminoflunitrazepam | 3 | 51.5 | 3 | 51.5 |
| Alprazolam | 5 | 23.1 | 10 | 31.3 |
| Chlordiazepoxide | 5 | 313.1 | 10 | 511.3 |
| Clonazepam | 10 | 97.5 | 10 | 97.5 |
| Codeine | 5 | 69.8 | 10 | 89.3 |
| Desalkylflurazepam | 5 | 60.9 | 10 | 101.6 |
| Diazepam | 5 | 291.1 | 10 | 432 |
| Dihydrocodeine | 5 | 57.9 | 10 | 81.9 |
| Fentanyl | 0.5 | 142.2 | 1 | 206.6 |
| Flunitrazepam | 3 | 20.5 | 3 | 20.5 |
| Hydrocodone | 1 | 43.4 | 10 | 127.4 |
| Hydromorphone | 3 | 65.6 | 3 | 65.6 |
| Lorazepam | 30 | 50.5 | 30 | 50.5 |
| Methadone | 5 | 131.4 | 10 | 216.3 |
| Midazolam | 1 | 79.8 | 10 | 299.4 |
| Morphine | 5 | 143.6 | 10 | 213.2 |
| Nordiazepam | 5 | 137.7 | 10 | 169.2 |
| Oxazepam | 5 | 36 | 10 | 63.6 |
| Oxycodone | 5 | 90 | 10 | 163.7 |
| Oxymorphone | 5 | 50.9 | 5 | 50.9 |
| Temazepam | 5 | 170.6 | 10 | 243.8 |
| Zaleplon | 10 | 132.7 | 10 | 132.7 |
| Zolpidem | 1 | 176.8 | 10 | 533.3 |
| Zopiclone | 5 | 206.5 | 10 | 317.3 |

*Conclusion*

The method demonstrated suitable LODs for the compounds validated.

**Sensitivity—Limit of Quantitation (LOQ)**

The LOQ was verified by two replicates of a standard run in five separate extractions prepared in a mixture of whole blood containing four different sources. All compounds had good chromatographic peak shape at the LOQ. All signal-to-noise ratios were greater than 31:1 for those compounds validated for quantitative analysis. MRM ion ratios were within 20% and retention times were within 0.1 minutes compared to the average of all calibrators used (specific to each compound). All quantitative results were within ± 20% of their prepared concentration.

| Analyte | Validated LOQ (ng/mL) | Lowest Signal to Noise at Validated LOQ |
|---|---|---|
| 6-MAM | 5 | 205.6 |
| 7-Aminoflunitrazepam | 3 | 51.5 |
| Alprazolam | 10 | 31.3 |
| Chlordiazepoxide | 50 | 1100.7 |
| Clonazepam | 10 | 97.5 |
| Codeine | 10 | 89.3 |
| Desalkylflurazepam | 10 | 101.6 |
| Diazepam | 10 | 432 |
| Dihydrocodeine | N/A | 0 |
| Fentanyl | 1 | 206.6 |
| Flunitrazepam | N/A | 0 |
| Hydrocodone | 10 | 127.4 |
| Hydromorphone | 3 | 65.6 |
| Lorazepam | 30 | 50.5 |
| Methadone | 50 | 336.1 |
| Midazolam | 10 | 299.4 |
| Morphine | 10 | 213.2 |
| Nordiazepam | 10 | 169.2 |
| Oxazepam | 10 | 63.6 |
| Oxycodone | 10 | 163.7 |
| Oxymorphone | 5 | 50.9 |
| Temazepam | 30 | 467.1 |
| Zaleplon | 10 | 132.7 |
| Zolpidem | 10 | 533.3 |
| Zopiclone | 30 | 609.8 |

*Conclusion*

The method demonstrated suitable LOQs for those compounds validated for quantitative analysis. Dihydrocodeine and flunitrazepam were validated for qualitative identification only.

**Repeatability—Bias (Accuracy)**

Standards prepared at least at 3 different concentrations—low, medium, and high levels meeting validation requirements were evaluated for each compound. Three replicates from five separate extractions were evaluated for each level with the exception of the 1/10 ng/mL control which had two replicates from five separate extractions. The bias was within ± 20% of the prepared concentration for all those compounds validated for quantitative analysis above their respective LOQs. The control levels evaluated for each compound are outlined below followed by the results of the bias evaluation.

| Analyte | Control Levels | 1:10 Dilution Control |
|---|---|---|
| 6-MAM | 5,20,40 | 200 |
| 7-Aminoflunitrazepam | 3,5,20,40 | 200 |
| Alprazolam | 10,30,50,200,400 | 2000 |
| Chlordiazepoxide | 50,200,400 | 2000 |
| Clonazepam | 10,30,50,200,400 | 2000 |
| Codeine | 10,30,50,200,400 | 2000 |
| Desalkylflurazepam | 10,30,50,200,400 | 2000 |
| Diazepam | 10,30,50,200,400 | 2000 |
| Dihydrocodeine | 10,30,50,200,400 | 2000 |
| Fentanyl | 1,3,5,20,40 | 200 |
| Flunitrazepam | 3,5,20,40 | 200 |
| Hydrocodone | 10,30,50,200,400 | 2000 |
| Hydromorphone | 3,5,20,40 | 200 |
| Lorazepam | 30,50,200,400 | 2000 |
| Methadone | 50,200,400 | 2000 |
| Midazolam | 10,30,50,200,400 | 2000 |
| Morphine | 10,30,50,200,400 | 2000 |
| Nordiazepam | 10,30,50,200,400 | 2000 |
| Oxazepam | 10,30,50,200,400 | 2000 |
| Oxycodone | 10,30,50,200,400 | 2000 |
| Oxymorphone | 5,20,40 | 200 |
| Temazepam | 30,50,200,400 | 2000 |
| Zaleplon | 10,30,50,200,400 | 2000 |
| Zolpidem | 10,30,50,200,400 | 2000 |
| Zopiclone | 30,50,200,400 | 2000 |

| Analyte | Bias (Accuracy) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1/10 ng/mL Control | | | | | 3/30 ng/mL Control | | | | |
| | Step 3 | Step 4 | Step 5 | Step 6 | Step 7 | Step 3 | Step 4 | Step 5 | Step 6 | Step 7 |
| 6-MAM | | | | | | | | | | |
| 7-Aminoflunitrazepam | | | | | | -3% | 3% | 7% | 1% | 3% |
| Alprazolam | 4% | 5% | 9% | 7% | 6% | -1% | 3% | 4% | -3% | 4% |
| Chlordiazepoxide | | | | | | | | | | |
| Clonazepam | -2% | 10% | -3% | -1% | 4% | 4% | 6% | 1% | -2% | -7% |
| Codeine | 6% | 1% | 2% | 4% | 7% | 4% | 0% | 7% | 5% | -1% |
| Desalkylflurazepam | -1% | 8% | 4% | 3% | -3% | -7% | 0% | -2% | 2% | 9% |
| Diazepam | -1% | 6% | 7% | 10% | -3% | 3% | 3% | 4% | 3% | -3% |
| Dihydrocodeine | | | | | | | | | | |
| Fentanyl | 0% | 10% | 5% | 0% | 0% | -6% | 0% | 1% | 0% | -2% |
| Flunitrazepam | | | | | | | | | | |
| Hydrocodone | 4% | 14% | 16% | -5% | -2% | -3% | -1% | -2% | 0% | -4% |
| Hydromorphone | | | | | | 3% | -8% | -3% | -4% | -3% |
| Lorazepam | | | | | | -6% | -8% | 9% | 6% | 4% |
| Methadone | | | | | | | | | | |
| Midazolam | 2% | 11% | 18% | 2% | -4% | 1% | 4% | 5% | -1% | 2% |
| Morphine | 9% | 16% | 8% | 8% | -4% | 3% | 3% | 1% | 1% | -3% |
| Nordiazepam | -3% | 6% | 1% | 6% | -3% | -1% | 3% | 0% | 5% | -5% |
| Oxazepam | 4% | 4% | 16% | 15% | 4% | 8% | 1% | 3% | 0% | 0% |
| Oxycodone | 4% | 4% | 0% | 3% | -4% | 1% | 2% | 2% | -4% | -1% |
| Oxymorphone | | | | | | | | | | |
| Temazepam | | | | | | -10% | -7% | -9% | -17% | -6% |
| Zaleplon | 3% | 11% | -19% | 8% | -4% | -3% | -1% | -2% | -4% | 5% |
| Zolpidem | -6% | 6% | 6% | 6% | 0% | 8% | 13% | 9% | 6% | 5% |
| Zopiclone | | | | | | -6% | 6% | 5% | 8% | 15% |

| Analyte | Bias (Accuracy) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 5/50 ng/mL Control | | | | | 20/200 ng/mL Control | | | | |
| | Step 3 | Step 4 | Step 5 | Step 6 | Step 7 | Step 3 | Step 4 | Step 5 | Step 6 | Step 7 |
| 6-MAM | 13% | 6% | 19% | 14% | 9% | 2% | 8% | 8% | 10% | 10% |
| 7-Aminoflunitrazepam | -5% | 0% | 3% | 5% | 3% | 6% | 4% | -3% | 5% | 7% |
| Alprazolam | -3% | -3% | 4% | -4% | 0% | 2% | -2% | 1% | -2% | 2% |
| Chlordiazepoxide | -5% | -2% | -2% | 8% | 14% | 11% | 0% | 2% | 3% | 3% |
| Clonazepam | 2% | -6% | -5% | -4% | -7% | 10% | -4% | 0% | -4% | 9% |
| Codeine | -1% | 4% | 0% | 2% | 1% | 9% | 5% | 0% | 4% | 3% |
| Desalkylflurazepam | -5% | -2% | 2% | 0% | 4% | 2% | -1% | 2% | 3% | 8% |
| Diazepam | -5% | 0% | 3% | 0% | 5% | 2% | -4% | 4% | -3% | 4% |
| Dihydrocodeine | | | | | | | | | | |
| Fentanyl | -6% | -6% | 0% | -4% | -4% | 0% | -10% | 0% | -2% | 0% |
| Flunitrazepam | | | | | | | | | | |
| Hydrocodone | -6% | -3% | 1% | -1% | -3% | -2% | -5% | 1% | -3% | 4% |
| Hydromorphone | -7% | -11% | 3% | -7% | -2% | -2% | -6% | 1% | -5% | 5% |
| Lorazepam | 1% | 0% | 8% | 2% | -3% | 5% | 2% | 4% | 4% | 1% |
| Methadone | 12% | -4% | 6% | 7% | 0% | 11% | 11% | 4% | -1% | 11% |
| Midazolam | 0% | 4% | 4% | 0% | 8% | 0% | -5% | 0% | -1% | 8% |
| Morphine | -3% | 3% | 1% | -3% | 6% | 2% | 3% | -4% | 1% | 3% |
| Nordiazepam | -4% | -2% | 2% | 4% | 0% | 2% | -1% | 5% | 2% | 4% |
| Oxazepam | 2% | -6% | 9% | -4% | 2% | 5% | -1% | -1% | -1% | 6% |
| Oxycodone | 1% | 0% | 5% | -1% | 5% | 1% | 2% | -2% | 1% | 9% |
| Oxymorphone | -3% | 10% | -4% | 1% | 0% | -2% | 3% | 0% | 7% | 6% |
| Temazepam | -7% | -2% | -1% | -14% | -3% | -1% | -3% | -1% | -9% | 2% |
| Zaleplon | 1% | 3% | 0% | -5% | 3% | 4% | 1% | -3% | 1% | 9% |
| Zolpidem | 8% | 8% | 9% | 11% | 8% | 1% | -2% | -3% | 1% | 4% |
| Zopiclone | -2% | 4% | -2% | -3% | 11% | 15% | 8% | 0% | -6% | -6% |

| Analyte | Bias (Accuracy) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 40/400 ng/mL Control | | | | | 200/2000 ng/mL 1:10 Dilution Control | | | | |
| | Step 3 | Step 4 | Step 5 | Step 6 | Step 7 | Step 3 | Step 4 | Step 5 | Step 6 | Step 7 |
| 6-MAM | 3% | 7% | 3% | 12% | 7% | 10% | 3% | 2% | 10% | 3% |
| 7-Aminoflunitrazepam | 5% | 3% | 4% | 0% | 7% | 3% | -6% | -13% | 2% | -1% |
| Alprazolam | -2% | -1% | 4% | -4% | 4% | 0% | -12% | -8% | -7% | 0% |
| Chlordiazepoxide | 5% | 0% | 5% | -2% | 0% | 6% | 0% | -4% | 0% | 5% |
| Clonazepam | 2% | 5% | 4% | 3% | -3% | -1% | -10% | -6% | -6% | 0% |
| Codeine | 5% | 1% | 13% | 3% | 5% | 2% | -3% | -11% | -1% | -10% |
| Desalkylflurazepam | 3% | 1% | 8% | 8% | 4% | 4% | -13% | -8% | -1% | 0% |
| Diazepam | -1% | -1% | 4% | -4% | 6% | -4% | -12% | -12% | -6% | 1% |
| Dihydrocodeine | | | | | | | | | | |
| Fentanyl | -1% | 2% | 5% | -6% | -3% | -5% | -14% | -11% | -8% | -9% |
| Flunitrazepam | | | | | | | | | | |
| Hydrocodone | -3% | 0% | 7% | -5% | -2% | -3% | -13% | -8% | -7% | -3% |
| Hydromorphone | -4% | -3% | 3% | -5% | 2% | -2% | -3% | -8% | -11% | -1% |
| Lorazepam | 2% | 5% | 9% | 3% | 3% | 7% | 3% | -10% | -1% | 5% |
| Methadone | 4% | 1% | 10% | -1% | 1% | -7% | -16% | -10% | -5% | -5% |
| Midazolam | -1% | 0% | 4% | -1% | 1% | -3% | -6% | -11% | -5% | -1% |
| Morphine | 1% | 4% | 8% | -1% | 0% | 3% | -7% | -1% | -5% | 3% |
| Nordiazepam | 9% | 4% | 6% | -5% | -7% | 1% | -7% | -7% | -1% | -1% |
| Oxazepam | 8% | 7% | 9% | -6% | 9% | -5% | -4% | -6% | -5% | -1% |
| Oxycodone | -2% | -7% | 7% | -2% | 4% | 3% | -7% | -8% | -10% | 1% |
| Oxymorphone | -3% | -4% | 12% | 10% | 5% | 1% | -1% | -1% | -1% | 2% |
| Temazepam | -7% | -8% | -1% | -5% | -6% | 2% | -9% | -11% | -9% | -4% |
| Zaleplon | -2% | 5% | 2% | 6% | 1% | -4% | -6% | -10% | 3% | 6% |
| Zolpidem | 1% | -1% | 10% | 1% | 3% | -3% | -8% | -7% | -4% | 0% |
| Zopiclone | 12% | 8% | 3% | 1% | -6% | 11% | 2% | -7% | -5% | -5% |

*Conclusion*

The method demonstrated an acceptable bias of within ± 20% for all those compounds validated for quantitative analysis.

**Repeatability—Within-run and between-run precision**

At least three levels were evaluated (the same standards that were used for the bias experiments). Three replicates from five separate extractions were evaluated for each level with the exception of the 1/10 ng/mL

control which had two replicates from five separate extractions. The CV was within 20% for all levels evaluated. See above for the control levels that were used for each compound.

## Within-run Precision

| Analyte | Within-Run Precision (%CV) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1/10 ng/mL Control | | | | | 3/30 ng/mL Control | | | | |
| | Step 3 | Step 4 | Step 5 | Step 6 | Step 7 | Step 3 | Step 4 | Step 5 | Step 6 | Step 7 |
| 6-MAM | | | | | | | | | | |
| 7-Aminoflunitrazepam | | | | | | 3% | 3% | 11% | 2% | 0% |
| Alprazolam | 2% | 8% | 8% | 2% | 0% | 5% | 5% | 8% | 2% | 3% |
| Chlordiazepoxide | | | | | | | | | | |
| Clonazepam | 1% | 4% | 12% | 2% | 3% | 6% | 2% | 10% | 9% | 4% |
| Codeine | 3% | 6% | 3% | 2% | 5% | 4% | 1% | 5% | 5% | 4% |
| Desalkylflurazepam | 1% | 4% | 0% | 4% | 4% | 5% | 4% | 6% | 4% | 4% |
| Diazepam | 4% | 3% | 5% | 3% | 1% | 4% | 2% | 10% | 1% | 6% |
| Dihydrocodeine | 4% | 1% | 0% | 1% | 3% | 2% | 4% | 7% | 6% | 4% |
| Fentanyl | 0% | 0% | 7% | 0% | 0% | 5% | 3% | 4% | 3% | 4% |
| Flunitrazepam | | | | | | 10% | 2% | 15% | 12% | 13% |
| Hydrocodone | 0% | 2% | 2% | 4% | 1% | 3% | 6% | 3% | 2% | 2% |
| Hydromorphone | | | | | | 3% | 6% | 9% | 2% | 10% |
| Lorazepam | | | | | | 2% | 3% | 7% | 2% | 5% |
| Methadone | | | | | | | | | | |
| Midazolam | 1% | 1% | 3% | 5% | 10% | 2% | 2% | 1% | 1% | 3% |
| Morphine | 1% | 4% | 4% | 7% | 2% | 3% | 1% | 3% | 2% | 2% |
| Nordiazepam | 1% | 1% | 6% | 4% | 4% | 5% | 1% | 9% | 3% | 9% |
| Oxazepam | 3% | 5% | 5% | 5% | 6% | 7% | 4% | 11% | 4% | 8% |
| Oxycodone | 6% | 12% | 3% | 5% | 16% | 2% | 0% | 5% | 6% | 5% |
| Oxymorphone | | | | | | | | | | |
| Temazepam | | | | | | 6% | 5% | 5% | 3% | 3% |
| Zaleplon | 17% | 1% | 1% | 1% | 1% | 4% | 6% | 6% | 6% | 12% |
| Zolpidem | 3% | 7% | 3% | 1% | 1% | 1% | 1% | 2% | 1% | 3% |
| Zopiclone | | | | | | 3% | 2% | 10% | 6% | 1% |

| Analyte | Within-Run Precision (%CV) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 5/50 ng/mL Control | | | | | 20/200 ng/mL Control | | | | |
| | Step 3 | Step 4 | Step 5 | Step 6 | Step 7 | Step 3 | Step 4 | Step 5 | Step 6 | Step 7 |
| 6-MAM | 1% | 3% | 1% | 2% | 8% | 3% | 1% | 8% | 2% | 1% |
| 7-Aminoflunitrazepam | 3% | 4% | 1% | 1% | 1% | 1% | 7% | 3% | 2% | 4% |
| Alprazolam | 4% | 1% | 5% | 4% | 2% | 7% | 8% | 1% | 2% | 3% |
| Chlordiazepoxide | 2% | 2% | 3% | 4% | 3% | 4% | 5% | 4% | 1% | 5% |
| Clonazepam | 1% | 5% | 3% | 5% | 5% | 5% | 7% | 6% | 2% | 3% |
| Codeine | 4% | 4% | 6% | 4% | 2% | 1% | 6% | 1% | 0% | 11% |
| Desalkylflurazepam | 7% | 4% | 2% | 1% | 9% | 3% | 5% | 5% | 3% | 1% |
| Diazepam | 3% | 2% | 8% | 2% | 4% | 1% | 9% | 3% | 3% | 4% |
| Dihydrocodeine | 1% | 5% | 1% | 5% | 2% | 1% | 6% | 3% | 3% | 2% |
| Fentanyl | 0% | 2% | 5% | 2% | 2% | 2% | 8% | 1% | 3% | 2% |
| Flunitrazepam | 8% | 13% | 11% | 7% | 3% | 2% | 4% | 3% | 3% | 6% |
| Hydrocodone | 3% | 5% | 2% | 4% | 0% | 3% | 6% | 7% | 1% | 5% |
| Hydromorphone | 4% | 6% | 3% | 1% | 4% | 2% | 3% | 5% | 9% | 3% |
| Lorazepam | 2% | 5% | 5% | 6% | 1% | 3% | 1% | 8% | 4% | 4% |
| Methadone | 7% | 2% | 7% | 2% | 7% | 4% | 4% | 1% | 2% | 4% |
| Midazolam | 3% | 3% | 2% | 1% | 1% | 2% | 3% | 3% | 1% | 2% |
| Morphine | 2% | 1% | 3% | 4% | 2% | 3% | 8% | 5% | 2% | 4% |
| Nordiazepam | 5% | 4% | 4% | 2% | 4% | 4% | 6% | 5% | 5% | 8% |
| Oxazepam | 6% | 4% | 2% | 5% | 5% | 4% | 10% | 3% | 5% | 5% |
| Oxycodone | 3% | 6% | 12% | 7% | 6% | 3% | 3% | 5% | 5% | 5% |
| Oxymorphone | 4% | 6% | 6% | 5% | 2% | 1% | 5% | 9% | 8% | 2% |
| Temazepam | 3% | 4% | 4% | 1% | 4% | 6% | 6% | 4% | 6% | 5% |
| Zaleplon | 3% | 2% | 2% | 9% | 2% | 2% | 7% | 1% | 3% | 3% |
| Zolpidem | 1% | 1% | 6% | 2% | 5% | 1% | 6% | 2% | 4% | 5% |
| Zopiclone | 8% | 5% | 3% | 5% | 9% | 2% | 6% | 5% | 3% | 2% |

| Analyte | Within-Run Precision (%CV) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 40/400 ng/mL Control | | | | | 200/2000 ng/mL 1:10 Dilution Control | | | | |
| | Step 3 | Step 4 | Step 5 | Step 6 | Step 7 | Step 3 | Step 4 | Step 5 | Step 6 | Step 7 |
| 6-MAM | 3% | 6% | 6% | 5% | 6% | 5% | 3% | 3% | 2% | 7% |
| 7-Aminoflunitrazepam | 0% | 1% | 3% | 4% | 2% | 7% | 4% | 2% | 3% | 2% |
| Alprazolam | 6% | 2% | 3% | 8% | 4% | 8% | 9% | 7% | 5% | 5% |
| Chlordiazepoxide | 2% | 3% | 4% | 4% | 2% | 5% | 2% | 5% | 3% | 1% |
| Clonazepam | 3% | 11% | 8% | 6% | 9% | 6% | 9% | 5% | 9% | 6% |
| Codeine | 6% | 2% | 4% | 5% | 7% | 5% | 6% | 2% | 2% | 3% |
| Desalkylflurazepam | 4% | 8% | 3% | 4% | 3% | 8% | 4% | 0% | 6% | 3% |
| Diazepam | 2% | 8% | 1% | 6% | 1% | 4% | 5% | 2% | 3% | 4% |
| Dihydrocodeine | 2% | 6% | 3% | 1% | 2% | 2% | 5% | 3% | 3% | 2% |
| Fentanyl | 4% | 3% | 2% | 4% | 1% | 5% | 3% | 1% | 3% | 3% |
| Flunitrazepam | 9% | 7% | 6% | 3% | 8% | 4% | 1% | 3% | 6% | 1% |
| Hydrocodone | 1% | 3% | 3% | 7% | 5% | 4% | 3% | 2% | 1% | 3% |
| Hydromorphone | 4% | 3% | 4% | 4% | 3% | 2% | 3% | 1% | 5% | 4% |
| Lorazepam | 6% | 13% | 5% | 4% | 6% | 2% | 7% | 2% | 5% | 5% |
| Methadone | 6% | 2% | 5% | 4% | 2% | 7% | 6% | 8% | 5% | 4% |
| Midazolam | 1% | 3% | 3% | 3% | 1% | 7% | 2% | 3% | 2% | 4% |
| Morphine | 1% | 5% | 1% | 4% | 2% | 9% | 2% | 3% | 5% | 4% |
| Nordiazepam | 4% | 8% | 1% | 6% | 6% | 4% | 3% | 1% | 4% | 7% |
| Oxazepam | 7% | 4% | 8% | 3% | 6% | 3% | 9% | 4% | 1% | 5% |
| Oxycodone | 4% | 3% | 4% | 5% | 7% | 4% | 5% | 4% | 1% | 7% |
| Oxymorphone | 3% | 6% | 6% | 4% | 4% | 8% | 2% | 3% | 6% | 3% |
| Temazepam | 4% | 4% | 3% | 4% | 10% | 6% | 4% | 2% | 8% | 9% |
| Zaleplon | 2% | 2% | 6% | 4% | 7% | 10% | 3% | 4% | 3% | 3% |
| Zolpidem | 2% | 4% | 2% | 5% | 2% | 7% | 3% | 4% | 1% | 4% |
| Zopiclone | 1% | 3% | 3% | 2% | 2% | 9% | 4% | 2% | 2% | 5% |

## Between-run Precision

| Analyte | Between-Run Precision (%CV) | | | | | |
|---|---|---|---|---|---|---|
| | 1/10 ng/mL (n = 10) | 3/30 ng/mL (n = 15) | 5/50 ng/mL (n = 15) | 20/200 ng/mL (n = 15) | 40/400 ng/mL (n = 15) | 200/2000 ng/mL (n = 15) |
| 6-MAM | | | 5% | 5% | 6% | 5% |
| 7-Aminoflunitrazepam | | 6% | 4% | 5% | 3% | 7% |
| Alprazolam | 4% | 5% | 4% | 5% | 5% | 8% |
| Chlordiazepoxide | | | 8% | 5% | 4% | 5% |
| Clonazepam | 7% | 7% | 5% | 7% | 7% | 7% |
| Codeine | 4% | 5% | 4% | 6% | 6% | 7% |
| Desalkylflurazepam | 4% | 7% | 6% | 4% | 5% | 8% |
| Diazepam | 6% | 5% | 5% | 5% | 5% | 7% |
| Dihydrocodeine | 4% | 5% | 4% | 3% | 4% | 6% |
| Fentanyl | 5% | 4% | 3% | 5% | 5% | 4% |
| Flunitrazepam | | 16% | 10% | 4% | 7% | 6% |
| Hydrocodone | 8% | 3% | 4% | 5% | 6% | 5% |
| Hydromorphone | | 7% | 6% | 6% | 5% | 5% |
| Lorazepam | | 8% | 5% | 4% | 7% | 7% |
| Methadone | | | 8% | 5% | 5% | 7% |
| Midazolam | 8% | 3% | 4% | 5% | 3% | 5% |
| Morphine | 7% | 3% | 4% | 5% | 4% | 6% |
| Nordiazepam | 5% | 6% | 5% | 5% | 8% | 5% |
| Oxazepam | 7% | 7% | 7% | 6% | 8% | 5% |
| Oxycodone | 8% | 4% | 7% | 5% | 6% | 7% |
| Oxymorphone | | | 7% | 6% | 8% | 4% |
| Temazepam | | 6% | 6% | 6% | 5% | 7% |
| Zaleplon | 12% | 7% | 5% | 5% | 5% | 8% |
| Zolpidem | 6% | 3% | 3% | 4% | 5% | 5% |
| Zopiclone | | 8% | 8% | 9% | 6% | 9% |

*Conclusion*

The method demonstrated acceptable within-run and between-run precision with all CVs within 20%.

**Reportable range**

The reportable range was determined after evaluating the calibration model and sensitivity of the assay. The reportable range was determined to at least encompass the concentrations outlined belowbut may extend beyond the ranges evaluated in this validation. Compounds meeting acceptable identification criteria with apparent concentrations outside of the range identified below may be reported. With the validated dilution factor of 1:10 the upper end of the reportable range may be extended to ten times the number reported below.

| Analyte | Reportable Range (ng/mL) |
|---|---|
| 6-MAM | 5-100 |
| 7-Aminoflunitrazepam | 3-100 |
| Alprazolam | 5-1000 |
| Chlordiazepoxide | 5-1000 |
| Clonazepam | 10-1000 |
| Codeine | 5-1000 |
| Desalkylflurazepam | 5-1000 |
| Diazepam | 5-1000 |
| Dihydrocodeine | 5-1000 |
| Fentanyl | 0.5-100 |
| Flunitrazepam | 3-100 |
| Hydrocodone | 1-1000 |
| Hydromorphone | 3-100 |
| Lorazepam | 30-1000 |
| Methadone | 5-1000 |
| Midazolam | 1-1000 |
| Morphine | 5-1000 |
| Nordiazepam | 5-1000 |
| Oxazepam | 5-1000 |
| Oxycodone | 5-1000 |
| Oxymorphone | 5-100 |
| Temazepam | 5-1000 |
| Zaleplon | 10-1000 |
| Zolpidem | 1-1000 |
| Zopiclone | 5-1000 |

*Conclusion*

The reportable range determined in validation is appropriate for the compounds included in this method.

**Dilution Integrity**

Dilution integrity was evaluated for a 1:10 dilution of a 200/2000 ng/mL control prepared in whole blood. All stated bias and precision criteria were acceptable using the dilution. The results are included with the bias and precision data above.

*Conclusion*

All compounds validated for quantitative analysis demonstrated acceptable bias and precision using a 1:10 dilution. A 1:10 dilution may be used in routine casework for those samples with concentrations that may be above the highest calibrator.

**Carryover**

The lack of carryover was determined by triplicate analyses on five different days. A blank matrix sample was analyte free when run after a standard prepared at 100/1000 ng/mL.

*Conclusion*

The method demonstrated a lack of carryover up to a concentration of 100/1000 ng/mL. Matrix or solvent blanks will be run prior to each case sample to demonstrate that carryover did not occur.

**Extract Stability**

Five replicates of controls were prepared at a low, medium, and high concentration. The extracts were combined and then divided into five different vials. A vial of each level was injected in triplicate on day 0. The other vials were stored on the instrument and re-injected on each subsequent day in triplicate. The response of each analyte, internal standard, or relative response must be within ± 20% of the response from day 0. If the response falls outside this range then the extract stability of the analyte was exceeded. Extract stability varied from 0 to 4 days depending on the drug.

| Analyte | Extract Stability (# of days after day of extraction) |
|---|---|
| 6-MAM | 0 |
| 7-Aminoflunitrazepam | 0 |
| Alprazolam | 4 |
| Chlordiazepoxide | 0 |
| Clonazepam | 0 |
| Codeine | 4 |
| Desalkylflurazepam | 0 |
| Diazepam | 0 |
| Dihydrocodeine | 4 |
| Fentanyl | 4 |
| Flunitrazepam | 4 |
| Hydrocodone | 3 |
| Hydromorphone | 1 |
| Lorazepam | 0 |
| Methadone | 4 |
| Midazolam | 0 |
| Morphine | 4 |
| Nordiazepam | 0 |
| Oxazepam | 0 |
| Oxycodone | 0 |
| Oxymorphone | 0 |
| Temazepam | 0 |
| Zaleplon | 4 |
| Zolpidem | 0 |
| Zopiclone | 4 |

*Conclusion*

The extract stability of many drugs was exceeded on day 1. It is recommended that extracts not be rerun in excess of 24 hours after extraction unless the rerun is for a drug that demonstrated suitable extract stability. For routine analysis the batch may be run twice, back to back, due to the limited stability of many drugs in the extract.

**Ruggedness/Robustness**

Validation studies were performed by 5 different analysts over multiple days and demonstrated repeatable results. Some studies had to be repeated for some drugs and are summarized below.

| Step (Data file) | Issue | Analyte | Resolution |
|---|---|---|---|
| 3 (Replicates01) | 1:10 dilution low for all analytes | All | It was determined that the preparation of the dilution stock needed to be modified by adding a pulse vortex step to ensure sufficient mixing of the spike into whole blood. Data was used from an extraction performed on a different day for all 3 replicates. |
| 4 (Replicates02) | 1:10 dilution quant value low for one replicate of hydromorphone | hydromorphone | Data was used from an extraction performed on a different day (Repeat2/Replicates05b) for all three hydromorphone 1:10 dilution control replicates. |
| 4 (Replicates02) | $r^2$ of oxymorphone curve < 0.99 | oxymorphone | Data was used from an extraction performed on a different day (Repeat2/Replicates05b) for all oxymorphone standards. |
| 4 (Replicates02) | 10ng/mL CON-1 for alprazolam above 120% | alprazolam | Data was used from an extraction performed on a different day (Repeat/Replicates05a) for both of the two alprazolam 10ng/mL CONS. |
| 5 (Replicates03) | 10ng/mL CON-2 for codeine at 12.3 ng/mL | codeine | Data was used from an extraction performed on a different day (Repeat/Replicates05a) for both of the two codeine 10ng/mL CONS. |
| 5 (Replicates03) | 10ng/mL CON-2 for zaleplon above 120% | zaleplon | Data was used from an extraction performed on a different day (Repeat/Replicates05a) for both of the two zaleplon 10ng/mL CONS. |
| 6 (Replicates04) | 5ng/mL CON-2 for oxymorphone MRM outside +/-20% | oxymorphone | Data was used from an extraction performed on a different day (Repeat/Replicates05a) for all three oxymorphone 5ng/mL CONS. |
| 6 (Replicates04) | 10ng/mL CON-2 for oxycodone above 120% | oxycodone | Data was used from an extraction performed on a different day (Repeat3/Replicates05c) for both of the two oxycodone 10ng/mL CONS. |
| 6 (Replicates04) | 400ng/mL CON-2 for zaleplon above 120% | zaleplon | Data was used from an extraction performed on a different day (Repeat/Replicates05a) for all of the zaleplon 400ng/mL CONS. |
| 7 (Replicates05) | 1:10 dilution quant value low for one replicate of lorazepam | lorazepam | Data was used from an extraction performed on a different day (Repeat2/Replicates05b) for all three lorazepam 1:10 dilution control replicates. |

*Conclusion*

Overall the method demonstrated acceptable robustness and yielded repeatable results.

**Case Sample Comparison**

Thirty case samples and three College of American Pathologists (CAP) proficiency samples that had been previously analyzed were reanalyzed by the method for a case comparison/crossover study. Five of the case samples and one CAP proficiency were negative for the target analytes by

the original methods and the BEN/OPI/Z Quant by LCMSMS method. There were only two drugs that could not be confirmed by LC-MSMS that were identified in the original analysis. Both of these samples, however were in storage for approximately a year between the original analysis and the LC-MSMS analysis. The drugs, diazepam and lorazepam, may have limited stability in blood specimens for such long periods of storage. The LOD for lorazepam is also slightly lower (5 ng/mL) for the original method compared to the LC-MSMS method (10 ng/mL). There were several compounds that were not able to be identified in the original analysis, but were determined to be present by LC-MSMS as a result of better sensitivity by the LC-MSMS method. In particular it was noted that several samples contained fentanyl that was not originally identified by screening with the blood base procedure. An ELISA screen for fentanyl will be evaluated to achieve more robust screening for blood samples for fentanyl. Any compounds that showed better sensitivity, etc. by either the original method or LC-MSMS method are highlighted in green in the table below. Overall there was good agreement of the qualitative results between the original methods and the LC-MSMS method, within the capabilities of each specific method.

There was very good agreement of the quantitative results (within ± 20%) for the majority of compounds in the majority of samples. There were seven results that exceeded –20%. They are highlighted in red in the table below. Three of those results were for nordiazepam and oxazepam in cases in which chlordiazepoxide was also present ([redacted] and [redacted]). Chlordiazepoxide is metabolized to norchlordiazepoxide and demoxepam and then further to nordiazepam and oxazepam. Therefore, all four compounds may be present in blood specimens after consumption of chlordiazepoxide. Demoxepam and to a lesser extent, norchlordiazepoxide, when present in high concentrations can result in falsely elevated levels of nordiazepam and oxazepam when measured by GC/MS due to breakdown of demoxepam and norchlordiazepoxide in the high temperature GC inlet as described by Joyce, et. al (7). The same effect is not observed when analysis by LC-MSMS is performed, as there is no high temperature zone prior to the mass spectrometer. These observations were confirmed based on experiments performed during this validation with GC/MS and LC-MSMS in which the positive control was analyzed both with and without the addition of 1 μg/mL of chlordiazepoxide, norchlordiazepoxide, and demoxepam. With the GC/MS analysis significantly elevated levels of nordiazepam were observed for the control containing demoxepam (88%). Significantly elevated levels of oxazepam were also observed by GC/MS for the control containing norchlordiazepoxide (21%) and demoxepam (212%). The positive control containing chlordiazepoxide, norchlordiazepoxide, and demoxepam also demonstrated a significant elevation of nordiazepam (456%) and oxazepam (46%) concentrations. When the controls were reinjected, all demonstrated the return of nordiazepam to expected levels and even greater elevation of oxazepam, indicating that if the extract remains on the autosampler for a longer period of time before injection, only the oxazepam levels will be elevated. Conversely if the extract is injected shortly after the extraction is complete elevated levels for both nordiazepam and oxazepam may be observed. Other case samples ([redacted], [redacted]) that did not contain chlordiazepoxide demonstrated good agreement of quantitative results for nordiazepam and oxazepam within 14%.

One case ([redacted]) containing hydromorphone demonstrated quantitative results 23.6% lower than the original analysis (6.8 compared to 8.9 ng/mL), but the sample was in storage for greater than one year between the original analysis and the LC-MSMS analysis and may have experienced some degradation of the drug over that time. Insufficient specimen volume

remained to re-extract the sample to examine this possibility. Another case sample ([redacted]) containing hydromorphone in which the original analysis and LC-MSMS analysis occurred within 1 day of each other demonstrated quantitative results for the LC-MSMS method within 3% of the original method.

One case ([redacted]) containing zolpidem demonstrated quantitative results 21.4% lower than the original analysis (570 compared to 725 ng/mL). This was however within the calculated uncertainty of measurement of the original analysis of +/- 22%. Both results were obtained with a dilution of the blood specimen (5x for the original analysis and 10x for the LC-MSMS analysis). This may have contributed to the overall difference in the quantitative results. Another case sample ([redacted]) containing zolpidem had comparable quantitative results within 3% of the original analysis.

One case ([redacted]) containing alprazolam demonstrated results 32.8% lower than the original analysis using the blood benzodiazepine quantitation method (43 compared to 64 ng/mL). The case was in storage for almost a year, so to rule out drug degradation over that period of time it was re-extracted using the blood alprazolam quantitation method with a 5x dilution and the results of 70 ng/mL were comparable to the original analysis. The sample was exhausted to complete this evaluation and therefore no further testing could be conducted to ascertain the reason for the lower quantitative results by LC-MSMS. Thirteen other blood cases containing alprazolam all had comparable quantitative results within +/- 20% of the original analysis.

One CAP proficiency sample (14CAP-03) containing lorazepam demonstrated results 27.3% lower than the original analysis (125 compared to 172 ng/mL). The sample was re-extracted by both the original method and the LC-MSMS method and demonstrated similar results of 170 and 127 ng/mL, respectively. The reason for the difference in quantitative results was not able to be ascertained and will be further examined when CAP releases the acceptable results for this sample. One case sample [redacted]) containing lorazepam had comparable quantitative results within 5% of the original analysis.

All of the results of the case comparison/crossover study are presented in the table below.

| Case # | Original Analysis | | | LC-MSMS Analysis | | | % Difference | Comments |
|---|---|---|---|---|---|---|---|---|
| | Drug | Concentration (ng/mL) | Date of Analysis | Drug | Concentration (ng/mL) | Date of Analysis | | |
| [redacted] | Zopiclone | | 8/21/2012 | Zopiclone | 215 | 3/31/2014 | | |
| | Citalopram | | | Not target | | | | |
| | CE | < 50 | 3/4/2013 | Not target | | | | |
| | BE | 280 | 3/4/2013 | Not target | | | | |
| [redacted] | | | | Alprazolam | < 10 | 3/31/2014 | | Below 50ng/mL LOD of Blood Benzo |
| | Midazolam | 67 | 3/4/2013 | Midazolam | 76 | 3/31/2014 | 13.43% | |
| | Hydromorphone | 8.9 | 3/13/2013 | Hydromorphone | 6.8 | 3/31/2014 | -23.60% | Greater than 1 year in storage |
| [redacted] | ND | | 2/25/2013 | ND | | 2/20/2014 | | |
| | | | | | | | | 10x dilution on 2/28/14 LC-MSMS |
| | Diazepam | < 20 | 3/19/2013 | Diazepam | ND | 2/20/2014 | | ND 2/28/14 LC-MSMS |
| | Nordiazepam | 539 | 3/19/2013 | Nordiazepam | 497 | 2/20/2014 | -7.79% | 380 2/28/14 LC-MSMS |
| | Oxazepam | 240 | 3/19/2013 | Oxazepam | < 10 | 2/20/2014 | | ND 2/28/14 LC-MSMS |
| | Clonazepam | < 20 | 3/19/2013 | Clonazepam | 17 | 2/20/2014 | | < 100 2/28/14 LC-MSMS |
| | Chlordiazepoxide | | 3/21/2013 | Chlordiazepoxide | > 500 | 2/20/2014 | | 1160 2/28/14 LC-MSMS |
| [redacted] | Oxycodone | 110 | 3/20/2013 | Oxycodone | 112 | 2/20/2014 | 1.82% | < 100 2/28/14 LC-MSMS |
| | | | | Oxymorphone | < 5 | 2/20/2014 | | << Oxymorphone, OPI-OX not reported |
| | | | | Codeine | < 10 | 2/20/2014 | | OPI-OX LOD = 10 |
| | | | | | | | | Chlordiazepoxide metabolites break down in GC Inlet - can elevate Nordiazepam, Oxazepam levels |
| [redacted] | Citalopram | | 3/21/2013 | Not target | | | | |
| | Zolpidem | 267 | 3/21/2013 | Zolpidem | 273 | 2/20/2014 | 2.25% | 250 on 3/24/14 LC-MSMS (10x) |
| | Cocaine | < 50 | 5/23/2013 | Not target | | | | |
| | CE | < 50 | 5/23/2013 | Not target | | | | |
| | BE | 600 | 5/23/2013 | Not target | | | | |
| [redacted] | Alprazolam | 64 | 5/23/2013 | Alprazolam | 43 | 3/21/2014 | -32.81% | 3/24/14 ALP LLE (5x) = 70 |
| | THC | 3.6 | 5/3/2013 | Not target | | | | |
| | THCA | 9.2 | 5/3/2013 | Not target | | | | |

| Case # | Original Analysis | | | LC-MSMS Analysis | | | % Difference | Comments |
|---|---|---|---|---|---|---|---|---|
| | Drug | Concentration (ng/mL) | Date of Analysis | Drug | Concentration (ng/mL) | Date of Analysis | | |
| | Alprazolam | 30 | 2/27/2014 | Alprazolam | 34 | 2/25/2014 | 13.33% | 32 on 3/13/14 LC-MSMS |
| [redacted] | Hydromorphone | 8.2 | 2/26/2014 | Hydromorphone | 8.0 | 2/25/2014 | -2.44% | 9.0 on 3/13/14 LC-MSMS |
| | Zolpidem | 725 | 2/26/2014 | Zolpidem | 570 | 3/13/2014 | -21.38% | UOM = +/- 22% for Blood Base |
| | Chlordiazepoxide | | 9/18/2013 | Chlordiazepoxide | 1060 | 2/25/2014 | | 730 on 3/13/14 LC-MSMS |
| | Nordiazepam | 92 | 9/11/2013 | Nordiazepam | 64 | 2/25/2014 | -30.43% | 59 on 3/13/14 LC-MSMS |
| | Oxazepam | 146 | 9/11/2013 | Oxazepam | < 10 | 2/25/2014 | | < 10 on 3/13/14 LC-MSMS |
| [redacted] | Clonazepam | < 20 | 9/11/2013 | Clonazepam | 10 | | | 11 on 3/13/14 LC-MSMS |
| | | | | | | | | Chlordiazepoxide metabolites break down in GC Inlet - can elevate Nordiazepam, Oxazepam levels |
| | Carisoprodol | 8300 | 9/18/2013 | Not target | | | | |
| [redacted] | Meprobamate | 23000 | 9/18/2013 | Not target | | | | |
| | Clonazepam | 54 | 9/11/2013 | Clonazepam | 46 | 2/25/2014 | -14.81% | 44 on 3/13/14 LC-MSMS |
| | Hydrocodone | < 10 | 9/12/2013 | Hydrocodone | < 10 | 2/25/2014 | | < 10 on 3/13/14 LC-MSMS |
| | Cocaine | < 50 | 11/21/2013 | Not target | | | | |
| | CE | < 50 | 11/21/2013 | Not target | | | | |
| [redacted] | BE | > 1000 | 11/21/2013 | Not target | | | | |
| | Alprazolam | 16 | 11/25/2013 | Alprazolam | 19 | 2/13/2014 | 18.75% | 16 on 2/28/14 LC-MSMS |
| | Hydrocodone | < 10 | 2/26/2014 | Hydrocodone | < 10 | 2/13/2014 | | < 10 on 2/28/14 LC-MSMS |
| [redacted] | ND | | 3/7/2013 | ND | | | | |
| | | | | Alprazolam | 8.5 | 3/21/2014 | | Below 50ng/mL LOD of Blood Benzo |
| [redacted] | Clonazepam | 62 | 3/14/2013 | Clonazepam | 57 | 3/21/2014 | -8.06% | |
| | THCA | 4.4 | 3/20/2013 | Not target | | | | |
| [redacted] | Lorazepam | < 20 | 5/2/2013 | ND | | | | Below 10ng/mL LOD of LC-MSMS |
| | Alprazolam | 55 | 2/27/2014 | Alprazolam | 57 | 2/25/2014 | 3.64% | 49 on 3/13/14 LC-MSMS |
| [redacted] | Alprazolam | 93 | 8/29/2013 | Alprazolam | 80 | 2/13/2014 | -13.98% | 92 on 2/28/14 LC-MSMS |
| | BE | 70 | 8/28/2013 | Not target | | | | |
| | Alprazolam | 35 | 9/26/2013 | Alprazolam | 39 | 2/13/2014 | 11.43% | 43 on 2/28/14 LC-MSMS |
| | Diazepam | 20 | 9/11/2013 | Diazepam | 18 | 2/13/2014 | -10.00% | 18 on 2/28/14 LC-MSMS |
| [redacted] | Nordiazepam | 30 | 9/11/2013 | Nordiazepam | 26 | 2/13/2014 | -13.33% | 26 on 2/28/14 LC-MSMS |
| | THC | < 2.5 | 9/12/2013 | Not target | | | | |
| | THCA | 75 | 9/12/2013 | Not target | | | | |
| | THC | < 2.5 | 11/21/2013 | Not target | | | | |
| | THCA | 32 | 11/21/2013 | Not target | | | | |
| | Diazepam | 107 | 11/25/2013 | Diazepam | 102 | 2/13/2014 | -4.67% | 101 on 2/28/14 LC-MSMS |
| | Nordiazepam | 316 | 11/25/2013 | Nordiazepam | 305 | 2/13/2014 | -3.48% | 279 on 2/28/14 LC-MSMS |
| [redacted] | Oxazepam | < 20 | 11/25/2013 | Oxazepam | 12 | 2/13/2014 | | 11 on 2/28/14 LC-MSMS |
| | | | | Temazepam | < 30 | 2/13/2014 | | Below 25ng/mL LOD of Blood Benzo |
| | Alprazolam | 168 | 11/25/2013 | Alprazolam | 171 | 2/13/2014 | 1.79% | 169 on 2/28/14 LC-MSMS |
| | Morphine | 38 | 11/25/2013 | Morphine | 39 | 2/13/2014 | 2.63% | 39 on 2/28/14 LC-MSMS |
| | Amitriptyline | 196 | 12/11/2013 | Not target | | | | |
| | Nortriptyline | | 12/11/2013 | Not target | | | | |
| | Cocaine | 88 | 11/18/2013 | Not target | | | | |
| [redacted] | BE | 3275 | 11/18/2013 | Not target | | | | |
| | Lorazepam | 47 | 11/25/2013 | Lorazepam | 49 | 2/13/2014 | 4.26% | |
| | Oxycodone | < 10 | 11/25/2013 | Oxycodone | < 10 | 2/13/2014 | | |
| | Alprazolam | 91 | 1/28/2014 | Alprazolam | 97 | 3/31/2014 | 6.59% | |
| [redacted] | Benzoylecgonine | 63 | 12/20/2013 | Not target | | | | |
| | THCA | < 2.5 | 1/14/2014 | Not target | | | | |
| | | | | Hydrocodone | < 10 | 3/31/2014 | | ELISA OPI/OXY Negative |
| | Alprazolam | 7.1 | 1/14/2014 | Alprazolam | < 10 | 2/20/2014 | | < 10 on 3/24/14 LC-MSMS |
| [redacted] | Clonazepam | 29 | 3/31/2014 | Clonazepam | 29 | 2/20/2014 | 0.00% | 28 on 3/24/14 LC-MSMS |
| | Fentanyl | 6.3 | 3/13/2014 | Fentanyl | 6.3 | 2/20/2014 | 0.00% | ND in blood base screen 5.7 on 3/24/14 LC-MSMS |
| | | | | Morphine | < 10 | 2/20/2014 | | ELISA OPI Pos t ve, OPI-OX TMS Neg. 10 on 3/24/14 LC-MSMS |
| [redacted] | ND | | 1/7/2014 | ND | | 2/25/2014 | | ND on 3/13/14 LC-MSMS |
| | Alprazolam | 66 | 1/8/2014 | Alprazolam | 57 | 2/20/2014 | -13.64% | 71 on 3/24/14 LC-MSMS |
| [redacted] | THC | 3.1 | 1/14/2014 | Not target | | | | |
| | THCA | 40 | 1/14/2014 | Not target | | | | |
| [redacted] | Clonazepam | < 20 | 2/13/2014 | Clonazepam | 19 | 3/31/2014 | | |
| | Pentobarbital | | 3/10/2014 | Not target | | | | |
| | 6-MAM | 3.5 | 3/26/2014 | 6-MAM | < 5 | 3/27/2014 | | |
| [redacted] | Morphine | 79 | 3/26/2014 | Morphine | 89 | 3/27/2014 | 12.66% | |
| | | | | Codeine | < 10 | 3/27/2014 | | OPI-OX LOD = 10 |

| Case # | Original Analysis | | | LC-MSMS Analysis | | | % Difference | Comments |
|---|---|---|---|---|---|---|---|---|
| | Drug | Concentration (ng/mL) | Date of Analysis | Drug | Concentration (ng/mL) | Date of Analysis | | |
| | BEN/OPI/Z ND | | 3/6/2014 | ND | | 3/31/2014 | | |
| [redacted] | THC | 6.1 | 3/7/2014 | Not target | | | | |
| | THCA | 74 | 3/10/2014 | Not target | | | | |
| | Alprazolam | 41 | 3/10/2014 | Alprazolam | 38 | 3/20/2014 | -7.32% | |
| [redacted] | Morphine | < 10 | 3/26/2014 | Morphine | 10 | 3/20/2014 | | |
| | BE | 90 | 3/11/2014 | Not target | | | | |
| | Fentanyl | 5.0 | 4/10/2014 | Fentanyl | 4.0 | 3/20/2014 | -20.00% | ND in blood base screen |
| | Alprazolam | 190 | 4/1/2014 | Alprazolam | 154 | 4/1/2014 | -18.95% | |
| | Oxycodone | 126 | 3/26/2014 | Oxycodone | 120 | 4/1/2014 | -4.76% | |
| [redacted] | Oxymorphone | 2.5 | 3/26/2014 | Oxymorphone | < 5 | 4/1/2014 | | |
| | | | | Morphine | < 10 | 4/1/2014 | | << Morphine not reported OPI-OX |
| | | | | Nordiazepam | < 10 | 4/1/2014 | | << Nordiazepam not reported BBEN |
| | Cocaine | 104 | 3/20/2014 | Not target | | | | |
| | BE | > 1000 | 3/20/2014 | Not target | | | | |
| [redacted] | Alprazolam | 28 | 3/21/2014 | Alprazolam | 25 | 4/1/2014 | -10.71% | |
| | THC | < 2.5 | 3/25/2014 | Not target | | | | |
| | THCA | 8.3 | 3/25/2014 | Not target | | | | |
| [redacted] | Clonazepam | 46 | 1/30/2014 | Clonazepam | 39 | 2/28/2014 | -15.22% | |
| | Morphine | 85 | 2/26/2014 | Morphine | 93 | 2/28/2014 | 9.41% | |
| [redacted] | Alprazolam | 77 | 3/10/2014 | Alprazolam | 73 | 4/1/2014 | -5.19% | |
| | THCA | < 2.5 | 3/10/2014 | Not target | | | | |
| | Lorazepam | 172 | 3/21/2014 | Lorazepam | 125 | 3/21/2014 | -27.33% | LC-MSMS on 4/1/14 = 127 |
| 14CAP-03 | Meperidine | 4580 | 3/27/2014 | Not target | | | | |
| | Normeperidine | 4040 | 3/27/2014 | Not target | | | | |
| | BEN/OPI/Z ND | | | ND | | 3/24/2014 | | |
| | Cocaine | 373 | 3/20/2014 | Not target | | | | |
| 14-FTCA-1 | BE | 3206 | 3/21/2014 | Not target | | | | |
| | THC | 30 | 3/28/2014 | Not target | | | | |
| | THCA | 43 | 3/28/2014 | Not target | | | | |
| 14-FTCA-2 | Codeine | 262 | 3/26/2014 | Codeine | 245 | 3/24/2014 | -6.49% | |
| | Chlorpheniramine | 161 | 4/2/2014 | Not target | | | | |

*Conclusion*

Overall the method demonstrated good agreement for both qualitative and quantitative results when compared to methods currently in use for casework. Increased sensitivity and detection was demonstrated for several compounds, especially fentanyl.

**Uncertainty of Measurement**

An estimation of the uncertainty of measurement was determined for each compound that was validated for quantitative analysis according to the currently approved procedure within the toxicology unit. At least thirty replicates of the 20/200 ng/mL control performed by 5 different analysts were used in the estimation. See the uncertainty worksheets maintained on the network or PBSO portal for the results.

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Approved by:_____ Date:____4/7/14_____

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