# Evaluation of an LC-MS/MS Research Method for the Analysis of 33 Benzodiazepines and their Metabolites

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Application Note 593

## Key Words
TSQ Vantage, Transcend TLX-1, benzodiazepines

## Goal
The goal of this work was to evaluate the RECIPE® ClinMass® LC-MS/MS Complete Kit for research purposes using an online method analysis of 33 benzodiazepines and benzodiazepine metabolites in serum.

## Introduction
Benzodiazepines are prescribed for the management of anxiety, sleeping disorders, muscle spasms, and seizures. Benzodiazepines are widely viewed as safe drugs that have relatively few side effects. However, high dosages over prolonged periods can lead to tolerance, which leads to a loss of efficacy and/or physical and psychological dependence, resulting in severe withdrawal symptoms. Benzodiazepines can also be abused in cases of crime, suicide, and drug-facilitated sexual assault. While benzodiazepine intoxication alone is rarely fatal, concurrent use with alcohol or other drugs can be life-threatening. For all of these reasons, additional research into dosage and effects of benzodiazepines is necessary.

Benzodiazepines are active at very low concentrations and have short half lives; therefore, it is of great interest to clinical researchers and forensic toxicologists to simultaneously analyze benzodiazepines and their metabolites in biological samples. However, the analysis is not always easy because of very low blood concentrations and the complexity of detecting multiple drugs at the same time, especially in a biological matrix. Typically, benzodiazepines are quantified in serum.

In this work, the RECIPE kit for benzodiazepines analysis in serum was evaluated for research purposes using a Thermo Scientific™ Transcend™ TLX-1 system for performing both the online solid-phase extraction (SPE) and the chromatography. The Transcend system was coupled to a Thermo Scientific™ TSQ Vantage™ triple-stage quadrupole mass spectrometer for quantitative analysis. The RECIPE kit allows the analysis of 33 benzodiazepines and their metabolites in 12–15 minutes for research applications, such as those discussed in this application note. It includes the SPE column, analytical column, mobile phases, optimization mixtures, calibrators, quality controls, and an internal standards solution that integrates 20 deuterated internal standards. One kit is sufficient for up to 200 samples.

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## Experimental

The research method was applied as described in the RECIPE ClinMass LC-MS/MS Complete Kit instructions, with the exception of the loading flow rate, which is described in the HPLC method.

### Sample Preparation

As described in the kit instructions, 50 µL of each calibrator and quality control was vortexed for 10 s in a sample preparation tube with 50 µL of internal standards solution. The sample was then centrifuged for 10 min at 10,000 rpm, and 20 µL of the supernatant was injected into the LC-MS/MS system.

### HPLC Method

The kit includes an online solid phase extraction (SPE) column and an HPLC separation column that are integrated in a valve system to operate by column switching. A Transcend TLX-1 system was used to perform this column switching. The plumbing diagram is shown in Figure 1.

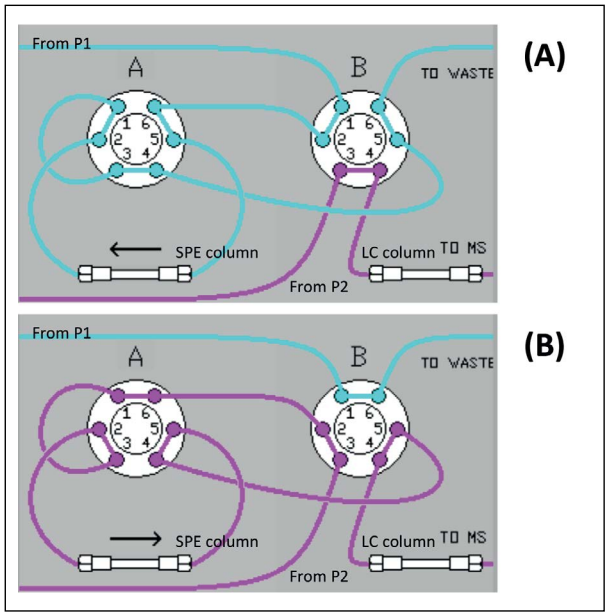


Figure 1. Plumbing of the Transcend system to perform column switching, with load (A) and inject (B) position, P1 is the loading pump and P2 the elution pump

The RECIPE kit was used as described in the instructions for this research purpose; however, the loading flow rate was 2 mL/min instead of the 5 mL/min described in the kit manual. This increased the time of analysis from 11 min to 12–15 min, but it did not impact the quality of the obtained data. In the first step, the valves are in load position (Figure 1A) where the sample is loaded onto the SPE column for the extraction of the analytes from the biological matrix. This step takes 1.9 min. In step two, the valves are switched to the inject position (Figure 1B) where the analytes extracted on the SPE column are eluted to the HPLC column by backflushing with mobile phase for 7.5 min. The analytes are then chromatographically separated with a gradient. For the last step, the valves are switched back to a loading position (Figure 1A) and both columns are re-equilibrated for the next injection. This step lasts 2.75 min.

### MS Method

Mass spectrometric analysis was performed using a TSQ Vantage triple-stage quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source. Source parameters are summarized in Table 1. MS analysis was performed in positive-ion selected-reaction monitoring (SRM) mode. The optimized SRM parameters for all the analytes and internal standards are presented in Table 2. The cycle time was set to 600 ms with a data acquisition window of 4 min for each analyte.

Table 1. Optimized source parameters

| Ion Source | APCI, Positive |
|---|---|
| Resolution Q1 and Q3 | 0.7 amu |
| Discharge Current | 5.0 µA |
| Vaporizer Temperature | 450 °C |
| Sheath Gas Pressure | 30 au |
| Aux Gas Pressure | 10 au |
| Capillary Temp | 250 °C |
| Collision Pressure | 1.5 mTorr |

## Results and Discussion

Calibration curves were plotted for each analyte with the three calibrators provided in the kit. The regression model for all the analytes was linear with different weighting according to the analyte. The limits of quantification (LOQ) were obtained by diluting the first calibrator with blank serum either two times or five times (the blank serum is the 0 calibrator solution). The LOQ were then determined as the lowest concentration for which the %RSD for 5 injections was less than 20% and the bias was less than 20%. The weighting, internal standards, correlation factor, and LOQ of the analytes are presented in Table 3. Examples of chromatograms obtained at the LOQ for some of the analyzed compounds are presented in Figure 2. As can be seen in Table 3, good linearity was obtained for all of the analytes in the concentration ranges of the kit calibrators. A blank sample injected after the upper limit of quantification (ULOQ) was used to evaluate carryover. The carryover was less than 10% of the signal obtained for the LOQ for all the analytes tested with this kit.

Table 2. SRM parameters used for the analysis

| Compound | Retention Time (minutes) | Precursor Ion | Product Ion | S-Lens | Collision Energy |
|---|---|---|---|---|---|
| 7-Aminoclonazepam | 4.56 | 286.1 | 222.2 | 114 | 24 |
| 7-Aminoflunitrazepam | 4.70 | 284.1 | 135.1 | 101 | 26 |
| 7-Aminonitrazepam | 4.56 | 252.1 | 121.1 | 96 | 26 |
| α-OH-Alprazolam | 6.20 | 325.1 | 297.1 | 102 | 24 |
| α-OH-Midazolam | 7.24 | 342.1 | 324.1 | 102 | 19 |
| α-OH-Triazolam | 5.95 | 359.1 | 331.0 | 101 | 23 |
| Alprazolam | 6.66 | 309.1 | 205.1 | 112 | 38 |
| Bromazepam | 5.73 | 316.0 | 182.1 | 100 | 31 |
| Chlordiazepoxide | 7.64 | 300.1 | 227.1 | 95 | 24 |
| Clobazam | 6.35 | 301.1 | 259.1 | 96 | 19 |
| Clonazepam | 6.10 | 316.1 | 270.1 | 114 | 23 |
| Demoxepam | 5.67 | 287.1 | 178.0 | 91 | 21 |
| Desalkylflurazepam | 7.02 | 289.1 | 140.1 | 123 | 28 |
| Desmethylflunitrazepam | 5.80 | 300.1 | 254.1 | 95 | 23 |
| Diazepam | 8.47 | 285.1 | 193.1 | 91 | 28 |
| Estazolam | 6.31 | 295.1 | 267.1 | 119 | 22 |
| Flunitrazepam | 6.20 | 314.1 | 268.1 | 89 | 25 |
| Flurazepam | 9.16 | 388.2 | 315.1 | 117 | 20 |
| Lorazepam | 6.67 | 323.0 | 277.1 | 100 | 21 |
| Lormetazepam | 7.32 | 335.1 | 289.1 | 88 | 21 |
| Medazepam | 10.57 | 271.1 | 207.1 | 86 | 26 |
| Midazolam | 8.41 | 326.1 | 291.1 | 129 | 25 |
| Nitrazepam | 6.20 | 282.1 | 236.1 | 95 | 23 |
| Norclobazam | 5.96 | 287.1 | 245.1 | 81 | 19 |
| Nordiazepam | 7.98 | 271.1 | 140.1 | 99 | 27 |
| Oxazepam | 6.76 | 287.1 | 241.1 | 106 | 21 |
| Prazepam | 10.03 | 325.1 | 271.1 | 100 | 21 |
| Temazepam | 7.12 | 301.1 | 255.1 | 96 | 22 |
| Tetrazepam | 9.96 | 289.1 | 225.2 | 117 | 28 |
| Trazodone | 9.34 | 372.2 | 148.1 | 115 | 32 |
| Triazolam | 6.49 | 343.1 | 308.1 | 146 | 25 |
| Zaleplone | 5.43 | 306.1 | 236.1 | 100 | 26 |
| Zolpidem | 7.10 | 308.2 | 235.2 | 116 | 33 |

Table 3. Calibration parameters and LOQ

| Compound | Internal Standard | Weighting | $R^2$ | LOQ (µg/L) |
|---|---|---|---|---|
| 7-Aminoclonazepam | 7-Aminoclonazepam-D4 | 1/X | 0.999 | 2.6 |
| 7-Aminoflunitrazepam | 7-Aminoflunitrazepam-D7 | $1/X^2$ | 0.995 | 2.6 |
| 7-Aminonitrazepam | 7-Aminoclonazepam-D4 | 1/X | 0.995 | 22.1 |
| α-OH-Alprazolam | α-OH-Alprazolam-D5 | 1/X | 0.993 | 2.7 |
| α-OH-Midazolam | α-OH-Midazolam-D4 | 1/X | 0.995 | 10.8 |
| α-OH-Triazolam | α-OH-Triazolam-D4 | 1/X | 0.997 | 4.6 |
| Alprazolam | Alprazolam-D5 | 1/X | 0.996 | 1.1 |
| Bromazepam | 7-Aminoflunitrazepam-D7 | 1/X | 0.997 | 15.8 |
| Chlordiazepoxide | Chlordiazepoxide-D5 | $1/X^2$ | 0.993 | 111.5 |
| Clobazam | Triazolam-D4 | $1/X^2$ | 0.992 | 9.5 |
| Clonazepam | Clonazepam-D4 | $1/X^2$ | 0.994 | 2.5 |
| Demoxepam | Clonazepam-D4 | 1/X | 0.997 | 216.0 |
| Desalkylflurazepam | Temazepam-D5 | $1/X^2$ | 0.993 | 9.7 |
| Desmethylflunitrazepam | Clonazepam-D4 | $1/X^2$ | 0.990 | 4.5 |
| Diazepam | Diazepam-D5 | $1/X^2$ | 0.993 | 20.4 |
| Estazolam | Estazolam-D5 | 1/X | 0.996 | 21.4 |
| Flunitrazepam | Flunitrazepam-D7 | 1/X | 0.990 | 5.2 |
| Flurazepam | Prazepam-D5 | Equal | 0.996 | 4.3 |
| Lorazepam | Lorazepam-D4 | $1/X^2$ | 0.996 | 20.6 |
| Lormetazepam | Nordiazepam-D5 | $1/X^2$ | 0.989 | 1.8 |
| Medazepam | Nordiazepam-D5 | Equal | 0.997 | 8.6 |
| Midazolam | Midazolam-D4 | Equal | 0.995 | 15.5 |
| Nitrazepam | Nitrazepam-D5 | $1/X^2$ | 0.997 | 10.5 |
| Norclobazam | α-OH-Triazolam-D4 | 1/X | 0.997 | 133.0 |
| Nordiazepam | Nordiazepam-D5 | 1/X | 0.997 | 16.7 |
| Oxazepam | Oxazepam-D5 | 1/X | 0.995 | 62.5 |
| Prazepam | Prazepam-D5 | 1/X | 0.999 | 41.7 |
| Temazepam | Temazepam-D5 | $1/X^2$ | 0.996 | 21.7 |
| Tetrazepam | Prazepam-D5 | Equal | 0.999 | 8.2 |
| Trazodone | Prazepam-D5 | 1/X | 0.997 | 82.5 |
| Triazolam | Triazolam-D4 | 1/X | 0.988 | 4.0 |
| Zaleplone | 7-Aminoflunitrazepam-D7 | 1/X | 0.999 | 4.2 |
| Zolpidem | Zolpidem-D6 | Equal | 0.998 | 21.2 |

The intraday and interday analytical accuracies and variability are presented in Tables 4 and 5, respectively. For each analyte, intraday variability and accuracy were determined by analyzing five samples at each QC level. For interday variability and accuracy, the process performed for intraday data was repeated on three different days. For the intraday study, the accuracy obtained was between 94% and 112% with a %RSD less than 12%. In the case of interday assays, the accuracy was between 93% and 111% with a %RSD less than 13%. The obtained results show a low variability for the two QC levels as well as good accuracy.

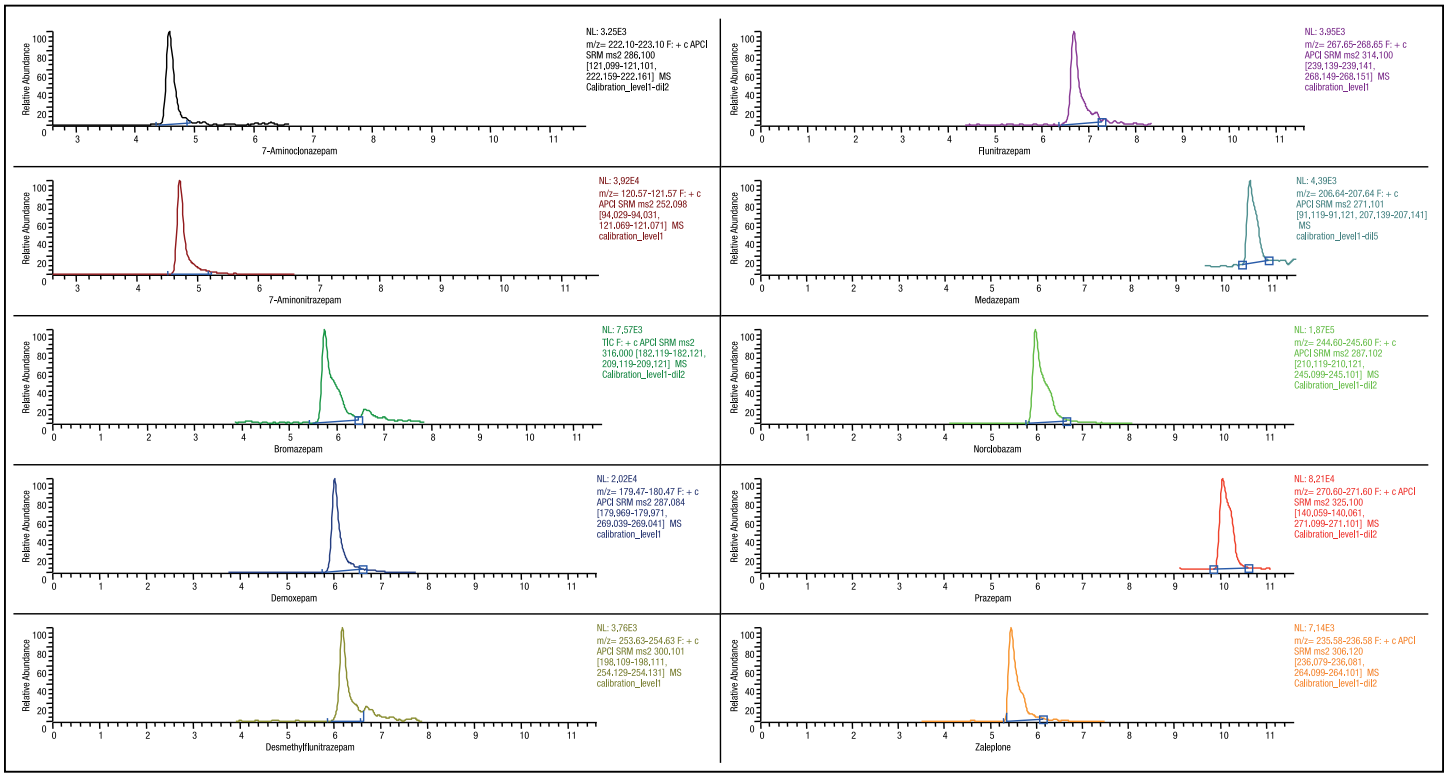


Figure 2. Chromatograms of the SRM transitions of some commonly found benzodiazepines at the LOQ

Table 4. Intraday repeatability and accuracy for QC1 and QC2

| | QC 1 | | | | QC 2 | | | |
|---|---|---|---|---|---|---|---|---|
| Compound | Specified Concentration | Measured Concentration | %RSD | %Accuracy | Specified Concentration | Measured Concentration | %RSD | %Accuracy |
| 7-Aminoclonazepam | 16.0 | 15.5 | 3.8 | 97 | 52.4 | 54.6 | 5.2 | 104 |
| 7-Aminoflunitrazepam | 16.6 | 16.8 | 8.6 | 101 | 54.5 | 55.9 | 3.0 | 103 |
| 7-Aminonitrazepam | 66.4 | 68.5 | 3.9 | 103 | 221 | 237 | 5.1 | 107 |
| α-OH-Alprazolam | 16.7 | 17.4 | 7.1 | 104 | 57 | 59.4 | 3.9 | 104 |
| α-OH-Midazolam | 64.6 | 65.4 | 3.8 | 101 | 203 | 217 | 4.1 | 107 |
| α-OH-Triazolam | 13.6 | 13.7 | 8.8 | 101 | 43.3 | 45.7 | 2.9 | 105 |
| Alprazolam | 15.4 | 15.7 | 9.2 | 102 | 55.5 | 55.2 | 5.8 | 99 |
| Bromazepam | 94.3 | 97.0 | 6.9 | 103 | 296 | 320 | 2.3 | 108 |
| Chlordiazepoxide | 621 | 602 | 3.1 | 97 | 2040 | 2150 | 3.3 | 105 |
| Clobazam | 143 | 144 | 7.7 | 101 | 491 | 489 | 4.4 | 100 |
| Clonazepam | 14.6 | 14.6 | 5.2 | 100 | 49.7 | 54.7 | 7.5 | 110 |
| Demoxepam | 682 | 673 | 5.5 | 99 | 2270 | 2350 | 4.4 | 103 |
| Desalkylflurazepam | 31.4 | 29.3 | 9.6 | 94 | 102 | 107 | 5.0 | 94 |
| Desmethylflunitrazepam | 15.2 | 14.4 | 4.6 | 95 | 51.1 | 54.2 | 3.8 | 106 |
| Diazepam | 292 | 297 | 3.4 | 102 | 949 | 941 | 5.2 | 99 |
| Estazolam | 132 | 127 | 2.4 | 96 | 441 | 447 | 6.6 | 101 |
| Flunitrazepam | 15.9 | 16.8 | 12.1 | 106 | 53.5 | 54.7 | 8.2 | 102 |
| Flurazepam | 25.9 | 26.9 | 5.4 | 104 | 86.2 | 93.0 | 4.7 | 108 |
| Lorazepam | 62.1 | 67.3 | 5.6 | 108 | 205 | 210 | 4.2 | 103 |
| Lormetazepam | 5.46 | 5.70 | 9.0 | 104 | 18.2 | 18.9 | 9.3 | 104 |
| Medazepam | 127 | 126 | 5.1 | 99 | 426 | 437 | 6.1 | 102 |
| Midazolam | 93.5 | 91.1 | 4.7 | 97 | 308 | 316 | 4.1 | 103 |
| Nitrazepam | 62.6 | 66.5 | 4.6 | 106 | 206 | 215 | 3.0 | 104 |
| Norclobazam | 835 | 811 | 3.2 | 97 | 2670 | 2770 | 3.8 | 104 |
| Nordiazepam | 239 | 245 | 4.2 | 103 | 821 | 804 | 4.3 | 98 |
| Oxazepam | 377 | 383 | 7.6 | 101 | 1240 | 1240 | 6.9 | 100 |
| Prazepam | 262 | 256 | 1.7 | 98 | 843 | 862 | 1.6 | 102 |
| Temazepam | 128 | 135 | 3.7 | 105 | 409 | 459 | 1.8 | 112 |
| Tetrazepam | 123 | 124 | 1.1 | 101 | 409 | 422 | 1.6 | 103 |
| Trazodone | 516 | 503 | 4.3 | 98 | 1630 | 1810 | 9.0 | 111 |
| Triazolam | 12.1 | 11.6 | 8.4 | 96 | 40.2 | 40.0 | 11.1 | 100 |
| Zaleplone | 26.8 | 27.3 | 8.5 | 102 | 88.6 | 94.1 | 5.6 | 106 |
| Zolpidem | 139 | 131 | 2.3 | 94 | 468 | 463 | 3.4 | 99 |

Table 5. Interday repeatability and accuracy for QC1 and QC2

| | QC 1 | | | | QC 2 | | | |
|---|---|---|---|---|---|---|---|---|
| Compound | Specified Concentration | Measured Concentration | %RSD | %Accuracy | Specified Concentration | Measured Concentration | %RSD | %Accuracy |
| 7-Aminoclonazepam | 16.0 | 16.7 | 6.3 | 104 | 52.4 | 53.9 | 5.3 | 103 |
| 7-Aminoflunitrazepam | 16.6 | 17.4 | 6.6 | 105 | 54.5 | 53.2 | 6.5 | 98 |
| 7-Aminonitrazepam | 66.4 | 70.7 | 5.8 | 107 | 221 | 224 | 6.1 | 101 |
| α-OH-Alprazolam | 16.7 | 17.8 | 7.4 | 107 | 57.0 | 56.8 | 5.7 | 100 |
| α-OH-Midazolam | 64.6 | 68.0 | 9.2 | 105 | 203 | 212 | 8.0 | 104 |
| α-OH-Triazolam | 13.6 | 13.4 | 6.3 | 98 | 43.3 | 43.5 | 5.3 | 100 |
| Alprazolam | 15.4 | 16.0 | 10.3 | 104 | 55.5 | 52.5 | 6.9 | 95 |
| Bromazepam | 94.3 | 91.9 | 12.9 | 97 | 296 | 310 | 7.9 | 105 |
| Chlordiazepoxide | 621 | 607 | 8.8 | 98 | 2040 | 2090 | 8.2 | 103 |
| Clobazam | 143 | 146 | 6.8 | 102 | 491 | 473 | 5.9 | 96 |
| Clonazepam | 14.6 | 15.0 | 5.6 | 103 | 49.7 | 52.7 | 5.9 | 106 |
| Demoxepam | 682 | 711 | 7.5 | 104 | 2270 | 2280 | 4.3 | 100 |
| Desalkylflurazepam | 31.4 | 29.5 | 10.1 | 94 | 102 | 102 | 7.3 | 100 |
| Desmethylflunitrazepam | 15.2 | 15.3 | 7.3 | 101 | 51.1 | 51.8 | 6.3 | 101 |
| Diazepam | 292 | 309 | 8.8 | 106 | 949 | 945 | 9.5 | 100 |
| Estazolam | 132 | 129 | 3.7 | 98 | 441 | 437 | 7.1 | 99 |
| Flunitrazepam | 15.9 | 17.4 | 9.5 | 109 | 53.5 | 52.6 | 8.7 | 98 |
| Flurazepam | 25.9 | 27.3 | 13.4 | 106 | 86.2 | 89.5 | 8.1 | 104 |
| Lorazepam | 62.1 | 66.0 | 6.1 | 106 | 205 | 200 | 5.7 | 97 |
| Lormetazepam | 5.46 | 6.00 | 11.0 | 111 | 18.2 | 18.8 | 8.8 | 103 |
| Medazepam | 127 | 131 | 6.9 | 103 | 426 | 437 | 12.5 | 102 |
| Midazolam | 93.5 | 89.0 | 4.4 | 95 | 308 | 307 | 4.3 | 100 |
| Nitrazepam | 62.6 | 69.3 | 6.9 | 111 | 206 | 203 | 6.7 | 99 |
| Norclobazam | 835 | 835 | 6.3 | 100 | 2670 | 2640 | 5.6 | 99 |
| Nordiazepam | 239 | 262 | 11.6 | 110 | 821 | 802 | 8.1 | 98 |
| Oxazepam | 377 | 387 | 9.3 | 103 | 1240 | 1250 | 6.5 | 101 |
| Prazepam | 262 | 265 | 3.7 | 101 | 843 | 845 | 4.1 | 100 |
| Temazepam | 128 | 134 | 5.8 | 105 | 409 | 437 | 6.2 | 107 |
| Tetrazepam | 123 | 127 | 4.4 | 103 | 409 | 416 | 7.5 | 102 |
| Trazodone | 516 | 501 | 6.6 | 97 | 1630 | 1750 | 11.6 | 107 |
| Triazolam | 12.1 | 11.7 | 6.1 | 97 | 40.2 | 39.7 | 6.8 | 99 |
| Zaleplone | 26.8 | 27.1 | 9.8 | 101 | 88.6 | 85.2 | 11.2 | 96 |
| Zolpidem | 139 | 130 | 7.8 | 93 | 468 | 450 | 8.0 | 96 |

## Conclusion

The analysis of benzodiazepines for research purposes presents a challenge due to their low concentrations, their short half life, and their diversity. In this work, the RECIPE ClinMass® complete kit for the quantification of 33 benzodiazepines and metabolites in serum was evaluated for research use with a TSQ Vantage triple quadrupole. The online SPE configuration was achieved by using a Transcend TLX-1 system. HPLC coupled to tandem mass spectrometry may be a suitable tool for the quantification of benzodiazepines in research applications since it can deal with the differences in polarities of the benzodiazepines and their metabolites. The use of SRM detection increases the analytical specificity needed to attain the low quantification ranges for these types of molecules. Finally, the use of LC-MS/MS simplifies sample preparation since the matrix clean-up is performed by online SPE.

This research method showed good results in terms of variability, precision, and dynamic range requirements for a clinical research method.

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