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Optimisation of a fast and easy quantification method for 54 benzodiazepines & Z-drugs, including 20 designer benzodiazepines, in plasma

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Aim

In the last decades, benzodiazepines/Z-drugs have earned their place in society, particularly in the treatment of anxiety and sleeping disorders. However their use comes with important side effects, withdrawal symptoms and dependency. [1] They are also increasingly being misused. Over the last few years growing numbers of benzodiazepine NPS have been reported to the EMCDDA (Figure 1.) They are predominantly detected in combination with alcohol and opioids, where the importance of their added suppressant effect is often overlooked. [2] This research aimed to create a cheap, easy and time-efficient method (in both sample preparation and instrumentation used) to accurately detect and quantify most of the commonly prescribed benzodiazepines, including 20 NPS, in plasma, for use in both a clinical and forensic toxicological setting.

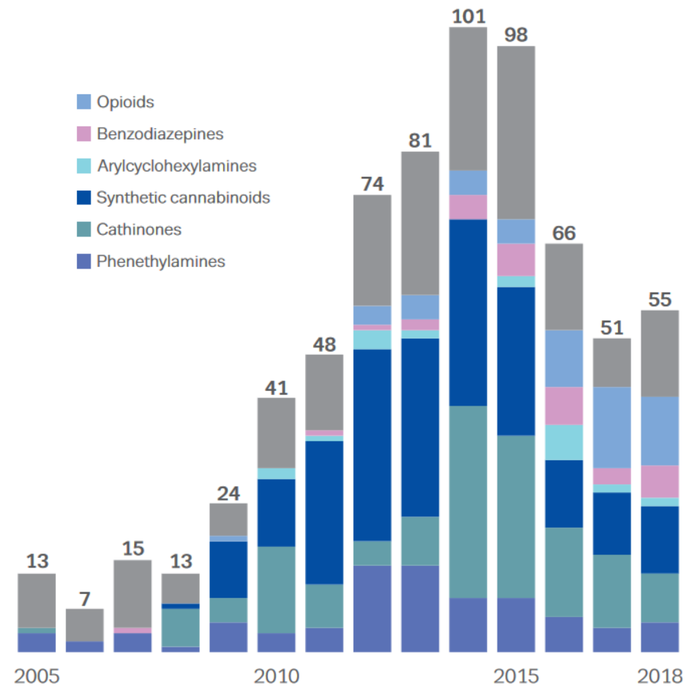

Figure 1. NPS notified to the EU Early Warning System for the first time, 2005-2018. [2]

Methodology

Selection of sample preparation method

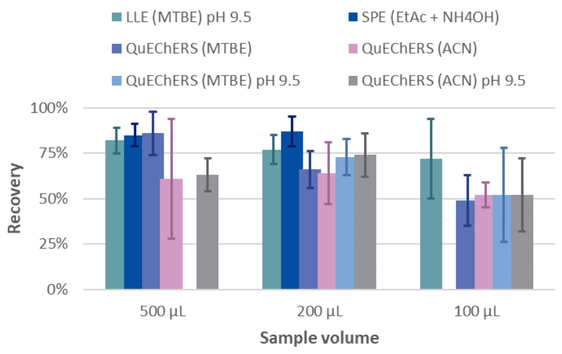

Figure 2. Comparison of the tested extraction protocols for nordazepam.

Plasma volumes 100, 200 and 500 µL were tested, 500 µL provided the required sensitivity. The QuEChERS initially gave promising results for the low sample volumes but the extraction efficiency and reproducibility were less than for the other methods. Implementing an additional clean-up step did not significantly improve the results. As both the LLE and SPE had similar recoveries (Figure 2), the LLE method was chosen due to time-efficiency and similarity with other methods. [3]

LC-QQQ settings

- Agilent 1200 series LC + 6460 QQQ
- Zorbax Eclipse Plus C8 column (2.1 x 150 mm, 3.5 µm) @ 40 °C
- MP A = H_2O + 0.1% FA (*V/V*); MP B = ACN:H_2O (9:1) + 0.1% FA (*V/V*)
- Start 5% B, to 95% B in 9 min + re-equilibration (total run time 12 min)
- dMRM mode, 54 target compounds, 20 deuterated internal standards

Validation data – results & discussion

Calibration range, accuracy & precision

Table 1. Calibration range, retention time, and within and between batch accuracy / precision for the compounds under investigation.

Compound	Calibration range (ng/mL)	Retention time (min)	Within batch (% ± RSD)	Between batch (% ± RSD)
3-OH-flubromazepam	2.5 - 1000	5.24	100 ± 9	103 ± 8
4-OH-midazolam	0.5 - 200	3.72	95 ± 6	105 ± 7
7-amino-clonazepam	2 - 800	2.85	103 ± 3	102 ± 7
7-amino-flunitrazepam	0.5 - 200	3.29	101 ± 5	102 ± 7
7-amino-nitrazepam	1 - 400	1.88	103 ± 4	102 ± 5
adinazolam	2.5 - 1000	3.63	100 ± 3	101 ± 6
alprazolam	1 - 400	5.34	93 ± 6	102 ± 9
α-OH-alprazolam	0.625 - 250	4.96	101 ± 6	99 ± 8
α-OH-midazolam	1.25 - 500	3.99	99 ± 5	105 ± 8
α-OH-triazolam	0.5 - 200	4.98	95 ± 11	104 ± 8
bentazepam	2.5 - 1000	3.89	101 ± 8	106 ± 7
bromazepam	2.5 - 1000	4.26	103 ± 3	101 ± 5
brotizolam	0.5 - 200	5.62	104 ± 5	103 ± 8
chlordiazepoxide	25 - 10000	3.55	106 ± 5	106 ± 5
clobazam	6.25 - 2500	5.94	96 ± 4	98 ± 5
clonazepam	2 - 800	5.38	92 ± 6	104 ± 6
clonazolam	2 - 800	5.02	98 ± 7	103 ± 8
cloniprazepam	0.5 - 200	7.01	99 ± 5	110 ± 6
clotiazepam	10 - 4000	5.85	97 ± 5	100 ± 7
cloxazolam	2.5 - 1000	3.03	98 ± 5	104 ± 6
delorazepam	2 - 800	5.87	100 ± 3	102 ± 6
deschloro-etizolam	1.25 - 500	4.90	108 ± 4	103 ± 6
diazepam	5 - 2000	6.12	104 ± 5	104 ± 3
diclazepam	1 - 400	6.47	104 ± 6	96 ± 7
ethyl loflazepate	2.5 - 1000	6.55	94 ± 7	100 ± 8
etizolam	1.25 - 500	5.67	102 ± 4	101 ± 4
flubromazepam	2.5 - 1000	5.73	97 ± 7	106 ± 7
flubromazolam	2 - 800	5.35	99 ± 5	101 ± 6
flunitrazepam	0.5 - 200	5.70	101 ± 7	100 ± 10
flurazepam	1 - 400	4.11	98 ± 3	99 ± 4
halazepam	10 - 4000	7.37	91 ± 5	101 ± 8
loprazolam	2 - 800	3.94	100 ± 6	106 ± 7
lorazepam	2 - 800	5.36	97 ± 17	97 ± 10
lormetazepam	0.5 - 200	5.95	104 ± 4	100 ± 5
meclonazepam	1 - 400	5.89	97 ± 7	100 ± 8
medazepam	10 - 4000	4.07	101 ± 6	101 ± 7
metizolam	1 - 400	5.47	103 ± 4	100 ± 6
midazolam	2.5 - 1000	4.01	100 ± 5	102 ± 4
nifoxipam	2.5 - 1000	4.59	102 ± 7	108 ± 7
nitrazepam	1 - 400	5.11	96 ± 9	105 ± 8
norclobazam	25 - 10000	5.37	111 ± 3	113 ± 3
nordazepam	10 - 4000	5.35	98 ± 5	102 ± 4
norflunitrazepam	0.5 - 200	5.15	104 ± 9	103 ± 9
norflurazepam	5 - 2000	5.61	101 ± 7	105 ± 5
oxazepam	10 - 4000	5.19	100 ± 4	99 ± 7
phenazepam	2 - 800	5.97	102 ± 5	102 ± 8
pivoxazepam	2.5 - 1000	7.87	68 ± 13	149 ± 18
prazepam	0.5 - 200	7.24	100 ± 2	102 ± 6
pyrazolam	2.5 - 1000	4.07	98 ± 6	103 ± 10
temazepam	10 - 4000	5.76	100 ± 4	103 ± 6
tetrazepam	6.25 - 2500	5.35	107 ± 6	105 ± 5
triazolam	0.5 - 200	5.47	105 ± 10	102 ± 7
zolpidem	5 - 2000	3.35	99 ± 3	99 ± 2
zopiclone	1 - 400	2.83	103 ± 3	104 ± 4

Table 1 gives an overview of the compounds under investigation and their basic analytical parameters. Calibration curves ranged over a factor 400. Both within and between batch accuracies and precisions were within the acceptability criteria (± 15%). For pivoxazepam problems were encountered with all quality control samples. Therefore it is included for qualitative analysis only.

A mixture of 20 labelled internal standards was added to all samples. Compounds for which a deuterated analogue was not available were linked to both a retention time and a structure matched labelled internal standard. Based on the overall validation data the internal standard that gave the most reliable result was chosen for the final method.

Matrix effect & recovery

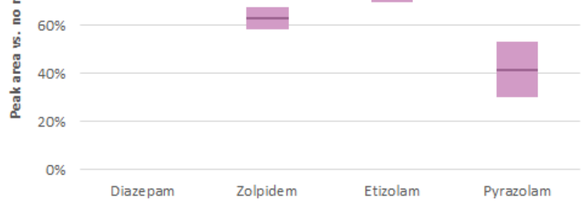

Figure 3. Absolute matrix effects and recoveries for selected compounds.

As with our other methods, no significant ion suppression or enhancement was noted (Figure 3). [3] Absolute matrix effects were ~101% ± 4%. Flunitrazepam-D_7 was suppressed by co-elution with lorazepam and was excluded.

Recoveries were ~79% ± 8% for benzodiazepines and ~ 68% ± 5% for Z-drugs. Pyrazolam showed poor but reproducible recoveries of ~42% ± 11%. No issues with the LLOQ or any other validation parameters were encountered.

Stability data

Benchtop stability was assessed for 3 hours at ambient temperature (Figure 4). The EMA validation criteria were met for all but 2 compounds. The concentration of cloxazolam decreased by 30%. The prodrug ethyl loflazepate may have been hydrolised to its active metabolite loflazepate, though reports state that this conversion takes place gradually. [4]

Autosampler stability was evaluated for 72 hours. The instrument room was kept to a constant temperature of 21 °C. Once extracted no further compound degradation was observed for the investigated time period.

Freeze-thaw stability was analysed for 3 consecutive cycles. Again ethyl loflazepate showed poor stability (1 cycle), in contrast to what has been published previously. [5] All other compounds were stable for a minimum of 2 cycles.

All compounds were stable for up to 1 month at -20 °C, many (~65%) for at least 3 months (Figure 5). Previous studies found excellent freeze-thaw and (in contrast to our results) benchtop stability for cloxazolam. [5] However no long-term stability data are available to compare our cloxazolam results to.

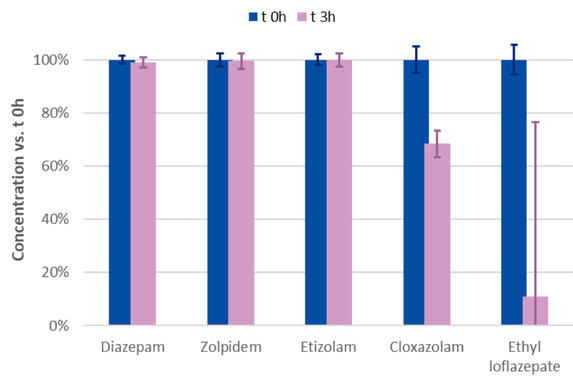

Figure 4. Benchtop stability for selected compounds.

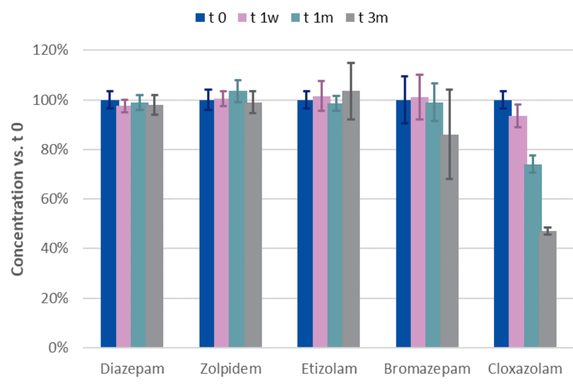

Figure 5. Long-term stability (-20 °C) for selected compounds.

Conclusion

Benzodiazepine drugs are widely used for their anxiolytic and hypnotic properties. In addition drug trends in Europe show a gradual increase in the detection of designer benzodiazepines mostly in combination with opioids. We present an analytical method that can be used for the identification and quantification of 54 benzodiazepines and Z-drugs. Keeping its application in both hospitals (for therapeutic drug monitoring) and forensic laboratories in mind, we opted to keep the sample preparation simple and the analytical instrumentation widely available. Different sample preparation methods were tested, with a liquid-liquid extraction using MTBE showing the best results.

The method was fully validated according to the European Medicines Agency's guidelines on bioanalytical method validation. Within and between batch accuracies and precisions were within the acceptability criteria for all compounds but pivoxazpam, due to a suspected issue with the quality control samples. As seen in previous methods the applied LLE method was effective in removing most matrix interferences. Extraction efficiency was acceptable with recoveries of ~70% - 80%. The compounds were stable both at room temperature and at -20 °C, with the exception of ethyl loflazepate which quickly broke down in samples when kept at room temperature.

References

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