Automated semi-quantitative screening of benzodiazepines and designer benzodiazepines in human serum using LC-ion trap-MS

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OVERVIEW

- Screening for benzodiazepines and designer benzodiazepines
- Semi-quantitative evaluation of LC-MS^n screening data
- LODs in the lower therapeutic range of most medical benzodiazepines

INTRODUCTION

In 2012 the group of New Psychoactive Substances (NPS) including numerous synthetic cannabinoids and designer stimulants ("bath salts") was extended by benzodiazepine-type compounds. At first, benzodiazepines like phenazepam and etizolam - which are still prescribed in some countries - were sold on the internet as recreational drugs. In the last years, the group of so-called designer benzodiazepines was enlarged by compounds that either are precursors (e.g. diclazepam) or active metabolites (e.g. norfludiazepam) of known benzodiazepines or combine structural properties of different classical benzodiazepines (e.g. flubromazolam). Considering the fact that patents and scientific literature describe the synthesis and detailed results of animal model studies for more than a hundred different benzodiazepines, it can be assumed that this sub-group of NPS will extend quickly in the future.

METHODS

Sample Preparation[1]: Alkaline liquid-liquid extraction

Extraction of 1 ml serum using 0.5 ml borate buffer (pH9) and 1.5 ml 1-chlorobutane after addition of three isotope labeled internal standard (IS). This sample preparation is identical to the one used for routine LC-MS^n screening, so extracts of real samples can be re-used.

LC - Settings

LC-System:	Dionex UltiMate 3000 LC-System
Eluent A:	Water, 2 mM ammonium formate, 0.1% formic acid, 1% acetonitrile
Eluent B:	Acetonitrile, 2 mM ammonium formate, 0.1% formic acid, 1% water
Column:	Acclaim® RSLC 120 C18 2,2 µm 120A 2.1x100 mm
Total flow:	500 µl/min
Injection vol.:	2 µl
Gradient:	0.0 to 0.2 min: 1% B 0.2 to 0.5 min: 1% B to 35% B, linear 0.5 to 6.0 min: 35% B to 40% B, linear 6.0 to 8.5 min: 40% B to 95% B, linear 8.5 to 11.0 min: 95% B 11.0 to 11.1 min: 95% B to 1% B, linear 11.1 to 13.0 min: 1% B

MS - Settings

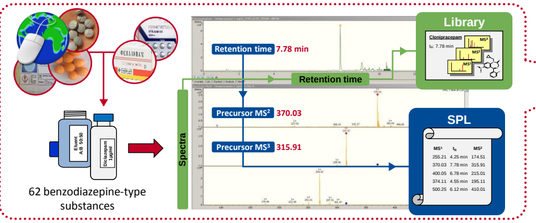
Bruker amaZon speed™ ion trap
- ESI source, positive mode
- UltraScan: 70 - 600 Da (32.500 Da/s)
- Auto MSn mode (n = 3)

Scheduled Precursor List to trigger data dependent acquisition of MS^2- and MS^3-spectra.

Data Evaluation and Reporting

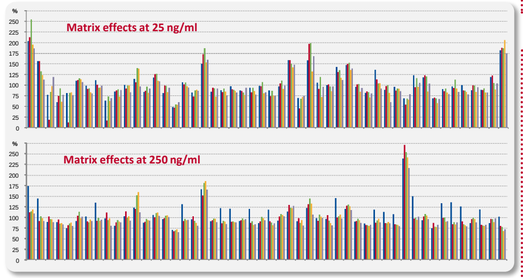
DataAnalysis 4.1 software package for automated data processing and result-reporting according to the Toxtyper-workflow[2].

Automated evaluation of quantitative results by a DataAnalysis (DA) script.



Limit of detection (LOD) was evaluated by fortifying 1 ml blank serum (n = 6) with different concentrations of benzodiazepines. LOD was set at the lowest concentration still leading to a positive identification by the DataAnalysis script in replicate determination.

Matrix effects (ME) and recovery (RE) were assessed according to Matuszewski et al.[3] using blank serum samples of five volunteers. For all three sets, two replica of a low and high concentration level were prepared and analyzed, subsequently.



Average ME varried between 53 and 211 % (SD: 3.0 - 33.6) for the low concentration levels (25 ng/ml, 50 ng/ml for compounds with high LOD) and between 68 and 244 % (SD: 1.9 - 24.4) at 250 ng/ml.
Evaluation of ME (110 %, SD: 11.4) and RE (0 %) for the high concentration of nitrazepam showed, that the non-satisfying LOD is probably caused by some kind of degradation when in contact with serum or the extraction solvents of the LLE.

Spectra recording and library building

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LC method development and optimization

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Evaluation of LOD

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Evaluation of linear range

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Evaluation of matrix effects and recovery

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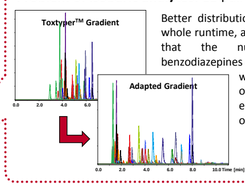
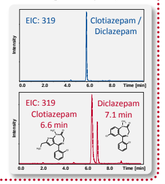
Evaluation of semi-quantitative results in serum

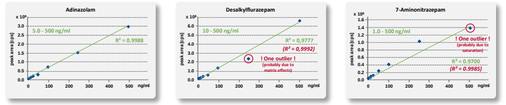
Problem of isobaric analytes: 12 pairs and one trio (not considering isotopes)



Better distribution of analytes over the whole runtime, also considering the fact that the number of designer benzodiazepines on the market probably will increase. Separation of isobaric analytes to enable proper integration of peaks in MS^1.

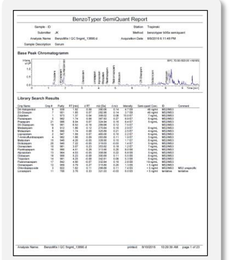


To set up the semi-quantitative part of the screening the linear range of each analyte has to be evaluated and defined in the DataAnalysis script. 1 ml pooled serum (n = 8) was spiked with 1.0 to 500 ng/ml of each compound.



To assess the linearity the peak area of the molecular ion ([M+H]$^+$) without normalization using peak area of internal standards (IS) was chosen. Suprisingly, using peak area ratios instead, led to lower R^2 values. Nevertheless, for semi-quantitative analysis of real samples use of IS is crucial.
The majority of compounds showed linear calibration curves from 5.0 ng/ml to 500 ng/ml as exemplified above for adinazolam. Desalkylflurazepam is shown as an example for good linearity over the whole concentration range but one outlier, probably due to matrix interference. For 20 analytes $R^2 > 0.99$ were observed, eliminating one outlier per substance this number went up to 40. For compounds like 7-aminonitrazepam the linear range was limited to 250 ng/ml.

A single-point calibrator (c = 50 ng/ml) in pooled serum, D5-Diazepam as IS and the linear ranges evaluated above were used for semi-quantitative screening of serum samples.
The peak area ratio of the molecular ion of the analyte and the IS was used for quantitation. Data evaluation was carried out automatically by the DA software. Positive findings below or above the linear range were reported as '< cal_{Low}' or '> cal_{High}', respectively. As usual, automatically generated screening results have to be revised for infrequent false positive findings by manual inspection of the spectra given in the report.
Semi-quantitative results in this preliminary study were found to vary between ± 20 and ± 50 % at the lower and upper end of the calibration range and ± 10 to ± 25 % at medium concentrations.



Report of a 5 ng/ml serum QC

RESULTS and DISCUSSION

The current spectral library contains 21 designer benzodiazepines and those prescription benzodiazepines most common in Germany, allowing the detection of 61 benzodiazepine-type substances and/or metabolites. The method can easily be extended once new compounds emerge on the drug market or according to specific needs of the user. The limit of detection was 5 ng/ml for the majority of the analytes, whereas nine compounds could only be detected at concentrations above 10 ng/ml. Nifoxipam, being highly instable in serum or during alkaline extraction, was the only compound that could not be detected at practically relevant concentrations in serum. Molecular ions of recently published metabolites or degradation products[4] could not be detected in MS^1.
For each analyte a linear calibration range (cal_{Low} to cal_{High}) was determined and calculated concentrations within this range are reported as semi-quantitative result in the automatically generated report. Due to data dependent acquisition of MS^n spectra, including active exclusion of precursors, in contrast to other MS/MS approaches only MS^1 full scan data is available for quantitation. This leads to a higher influence of coeluting compounds on peak shape and peak area, explaining the relatively high deviations seen in this study.
Nevertheless, this preliminary data demonstrates that semi-quantitative information can be obtained from ion trap screening data using single-point calibration. The used script automatically processes full scan data from a routine screening approach, so no modification to the acquisition method is required. Using customized calibration levels and suitable linear ranges, the obtained accuracy allows to distinguish therapeutic, sub-therapeutic and potentially toxic serum levels.
As mentioned above, use of internal standards is crucial for analyzing serum samples and confirmatory analysis using a validated quantitative approach is mandatory in forensic case work, of cause.

CONCLUSIONS

The presented method allows automated identification and semi-quantitative determination of 61 benzodiazepines, including 20 designer benzodiazepines. Limits of detection of the assay allow the detection of sub-therapeutic concentrations or concentrations in the low therapeutic range for the majority of medical benzodiazepines, making the screening applicable for clinical and forensic analysis. Semi-quantitative analysis enables a quick toxicological evaluation of the results and helps to decide on the analytical strategy in case work with limited sample volume available. Although this approach requires a more time consuming sample preparation when compared to routine immunoassays, unambiguous identification and semi-quantitative determination of compounds also offers more detailed information.

REFERENCES

[1] Demme et al.: *Poster presentation, TIAFT Meeting 2005*
[2] Meyer et al.: *Poster presentation (TP29), 61th ASMS Conference 2013*
[3] Matuszewski et al.: *Anal. Chem. 2003, 75, 3019-3030*
[4] Meyer et al.: *Anal Bioanal Chem 2016, 408:3571-3591*