Detection and semi-quantitative determination of designer benzodiazepines in serum using LC-MS^n

Benzodiazepines play an important role in forensic toxicology as they are widely prescribed in the treatment of psychiatric disorders and also used as drugs of abuse. In 2012 the group of New Psychoactive Substances (NPS) including numerous synthetic cannabinoids and designer stimulants ("bath salts") was extended by the inclusion of benzodiazepine-type compounds.

Introduction

Benzodiazepines such as phenazepam and etizolam - which are still prescribed in some countries - were initially available on the Internet providing an attractive and alternative source to prescription-only benzodiazepines. In the last years, the group of so-called designer benzodiazepines has been enlarged by compounds that are either precursors (e.g. diclazepam) or active metabolites (e.g. norfludiazepam) of known benzodiazepines or that combine structural properties of different classical benzodiazepines (e.g. flubromazolam). Since patents and scientific literature describe the synthesis, and detail 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

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future. In this study we describe the workflow to augment the Toxtyper™ library with new compounds of interest such as synthetic drugs. In addition, we show how semi-quantitative results may be obtained within the Toxtyper screening workflow.

Methods

Sample Preparation:

Serum samples were extracted using an alkaline liquid-liquid extraction currenty used for other routine LC-MS methods in the authors' laboratory[1]. D5-Diazepam, D4-Haloperidol and D3-Doxepin, 50 ng/mL each, were added as internal standards (IS) prior to extraction. 1 mL of serum was extracted using 0.5 mL of 1-clorobutane and 1.5 mL of borate buffer (pH 9). After 5 min of mixing, followed by a 10 min centrifugation at RT, the organic supernatant was transferred to an LC-vial, dried under a gentle stream of nitrogen (40°C) and the residue dissolved in 25 µL eluent A : B (50 : 50 (v/v)). This sample preparation is identical to that used for the Toxtyper™ screening, thus these extracts can be re-used for either comprehensive screening or semi-quantitative analysis of benzodiazepines.

LC-MS conditions:

For screening of benzodiazepines in serum, the Toxtyper LC gradient[2] was modified slightly to optimize the elution over the LC runtime, and to specifically decrease co-elution of isobaric compounds or co-elution of benzodiazepines with isobaric isotopes. As all benzodiazepines ionize in positive ESI mode, zero delay polarity switching of the Toxtyper approach was turned off and the ESI source was operated in positive mode only. The adapted LC-MS conditions are shown in Table 1 and Table 2.

Table 1: LC-conditions for separation of benzodiazepines.

LC-Conditions	
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
Analytical column	Acclaim® RSLC 120 C18 2,2 µm 120A 2.1x100 mm
Flow rate	500 µL/min
Injection volume	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

Table 2: MS-conditions for data dependent acquisition of spectra.

MS-Conditions	
MS-System	amaZon speed™ ion trap
Ion source	ESI source, positive mode
Scan mode	UltraScan: 70 - 600 Da at 32.500 Da/s
	Auto MS^n mode: n = 3
	Scheduled Precursor List to trigger MS^2- and MS^3- spectra

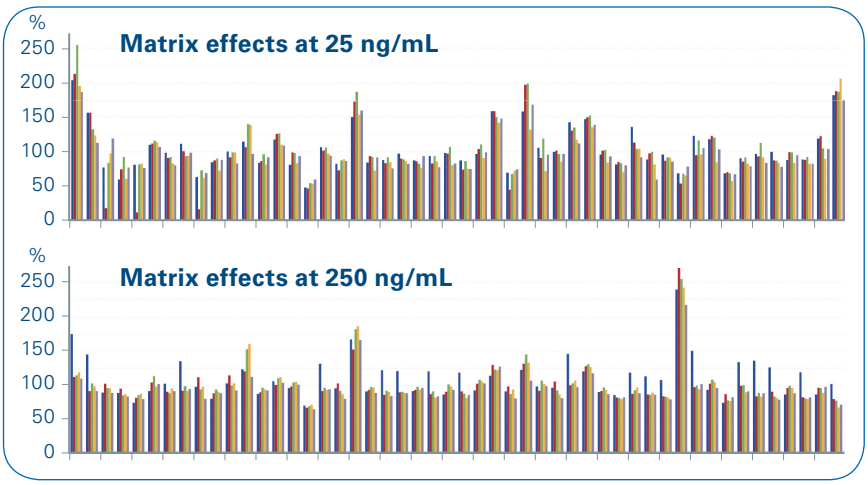


Figure 1: Evaluation of matrix effects in human serum (n = 5).

Spectra recording and building of the library:

For identification of new compounds of interest, spectral information from the Toxtyper library was extended by the addition of spectra of recently emerging designer benzodiazepines. Methanolic solutions of so called "research chemicals" were diluted in eluent (A : B = 50 : 50, v/v) and 1 µL was injected into the LC-MS-system using the LC conditions described above. To acquire library spectra, the most intense m/z ratio was isolated for generation of MS^2 or MS^3 spectra without a scheduled precursor list (SPL) and without 'active exclusion' of precursors. Within the Toxtyper workflow, the best spectrum was selected from the compound list, deconvoluted, converted to line spectrum, and checked according to the following criteria: mass difference

of the precursor ion in MS^1 and the calculated monoisotopic mass is within ± 0.25 amu; the intensity of the base peak in MS^2-stage is at least 10^5 counts, and the intensity of the base peak in MS^3-stage is at least 10^4 counts. Spectra matching these criteria were added to the library, including meta data such as empirical formula and associated structure.

Evaluation of limits of detection (LOD):

The limit of detection was evaluated by the addition of different concentrations of benzodiazepines to pooled blank serum aliquots (n = 6, 1 mL each) with different concentrations of benzodiazepines. The LOD of a substance was set at the lowest concentration resulting in a positive automatic identification in replicate determination (Table 3).

Evaluation of matrix effects:

Matrix effects (ME) and recovery (RE) were assessed according to Matuszewski et al.[3] using known blank serum samples from five volunteers. For all three sets, two replicates of a low and high concentration level were prepared and subsequently analyzed. Average ME varied 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 indicated that the non-satisfactory LOD is likely caused by compound degradation through contact with the serum or the extraction solvents of the LLE.

Table 3: Limits of detection (LOD) in pooled human serum.

Name	LOD [ng/mL]
2-OH-Ethylflurazepam	25
3-OH-Bromazepam	50
3-OH-Flubromazepam	25
3-OH-Phenazepam	25
7-Aminoclonazepam	10
7-Aminoflunitrazepam	1
7-Aminonitrazepam	1
Adinazolam	5
α-OH-Alprazolam	5
α-OH-Midazolam	5
α-OH-Triazolam	5
Alprazolam	1
Bromazepam	10
Chlordiazepoxide	5
Clobazam	1
Clonazepam	5
Clonazolam	5
Cloniprazepam	1
Clotiazepam	1
Delorazepam	5

Name	LOD [ng/mL]
Demoxepam	50
Desalkylflurazepam	10
Deschloroetizolam	1
Diazepam	5
Diclazepam	1
Etizolam	1
Flubromazepam	5
Flubromazolam	5
Fludiazepam	10
Flunitrazepam	1
Flunitrazolam	5
Flurazepam	1
Fonazepam	5
Loprazolam	1
Lorazepam	5
Lormetazepam	5
Meclonazepam	5
Medazepam	5
Metizolam	1
Midazolam	1

Name	LOD [ng/mL]
Norflunitrazepam	5
Nifoxipam	-
Nimetazepam	10
Nitrazepam	25
Nitrazolam	5
Norclobazam	10
Nordazepam	5
Oxazepam	25
Phenazepam	5
Prazepam	1
Pyrazolam	5
RO-5-4864	10
Temazepam	1
Tetrazepam	5
Triazolam	5
Zaleplone	5
Zolpidem	1
Zopiclone	5

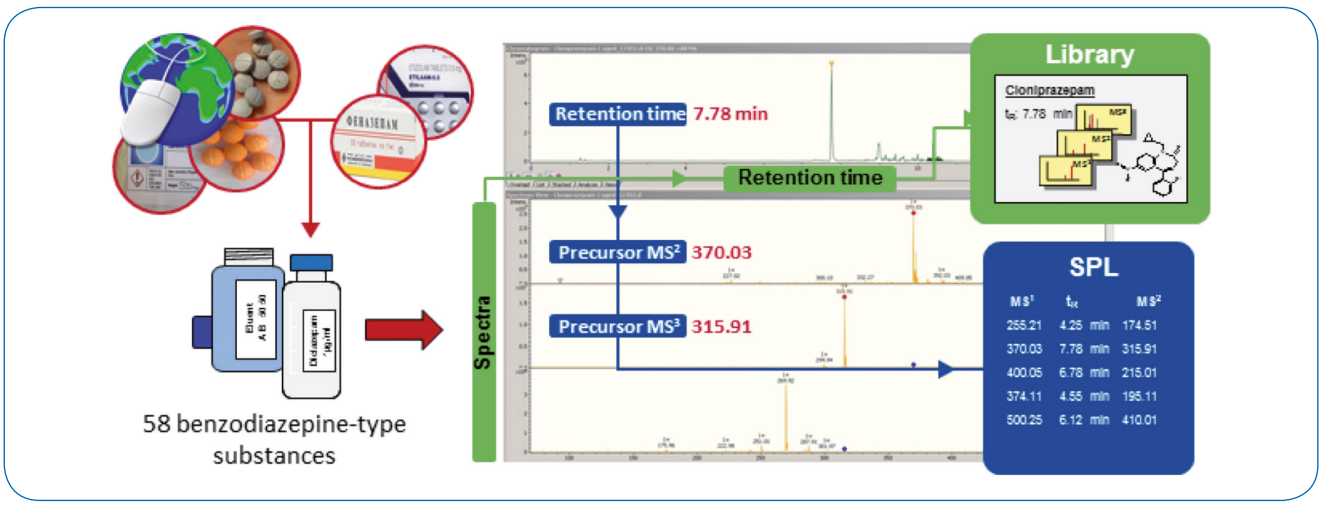


Figure 2: MS-conditions for data dependent acquisition of spectra.

Evaluation of linear concentration range:

To establish the semi-quantitative aspect of the target screening, the linear range of each analyte must be evaluated and defined. 1 mL pooled human serum (n = 8) was spiked with 1.0 to 500 ng/mL of each compound. To assess linearity, the peak area of each molecular ion ($[M+H]^+$) was used, without normalization using the peak area of internal standards (IS), as this approach resulted in higher R^2 values. Nevertheless, the use of internal standards is crucial for semi-quantitative analysis in analytical laboratory settings.

The majority of compounds showed linear calibration curves from 5.0 ng/mL to 500 ng/mL, as exemplified above for adinazolam (Figure 3, upper panel). Desalkylflurazepam (Figure 3, central panel) is shown as an example of good linearity over the whole concentration range including one outlier, likely due to matrix interference. For 20 of the analytes studied, R^2 values > 0.99 were observed, and this number increased to 38 with the elimination of one outlier per compound.

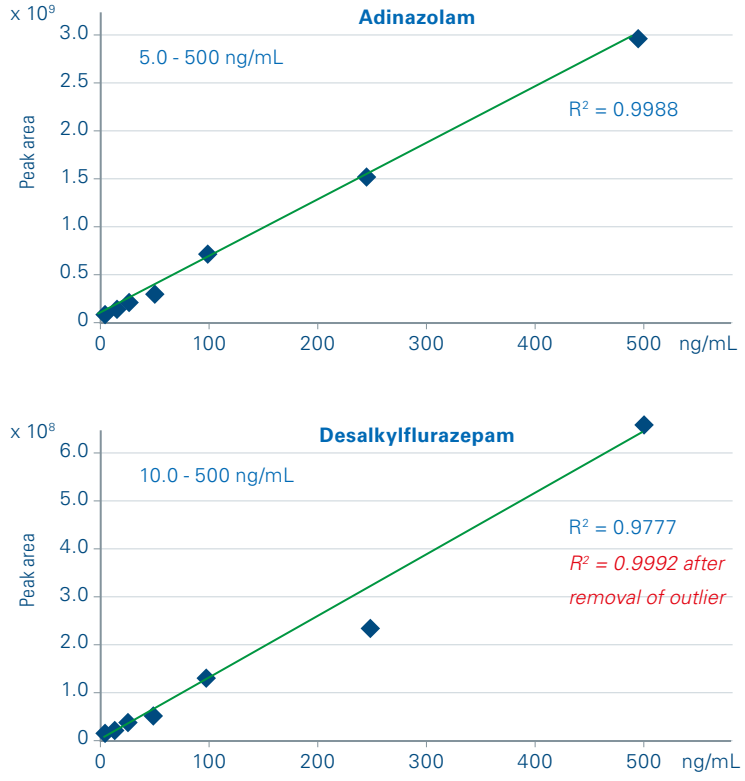
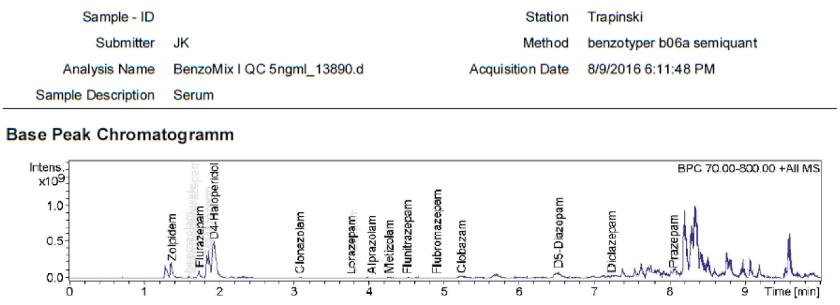


Figure 3: Examples of calibration curves without normalization using an isotope labeled standard.

Evaluation of semi-quantitative results in serum:

A single-point calibrator (c = 50 ng/mL) and an internal standard (D5-Diazepam) each in pooled serum, were used for semi-quantitative screening of serum samples. The peak area ratio of the molecular ion of the analyte and the internal standard was used for quantitation. Data evaluation was automatically carried out by the DataAnalysis software package. Positive findings below or above the linear range were reported as '< cal_{Low}' or '> cal_{High}', respectively. Following good laboratory practice, automatically generated screening results must be reviewed for infrequent false positives by manual inspection of the spectra available within the report.



Sample - ID		Station	Trapinski
Submitter	JK	Method	benzotyper b06a semiquant
Analysis Name	BenzoMix I QC 5ngml_13890.d	Acquisition Date	8/9/2016 6:11:48 PM
Sample Description	Serum		

Base Peak Chromatogramm

Library Search Results

Cmp Name	Cmp #	Purity	RT [min]	d RT	m/z [Da]	d m/z	Intensity	Semi-quant Conc.	ID	Comment
D4-Haloperidol	9	938	1.92	0.08	380.06	0.14	4.7 E8	48 ng/ml	MS2/MS3	
D3-Doxepin	7	951	1.85	0.07	282.96	0.14	3.7 E8	49 ng/ml	MS2/MS3	
Zolpidem	1	973	1.37	0.04	308.02	0.08	10.0 E7	7 ng/mL	MS2/MS3	
Flurazepam	5	992	1.74	0.08	387.93	0.27	8.9 E7	6 ng/mL	MS2/MS3	
Prazepam	21	996	8.04	0.07	324.94	0.16	8.4 E7	5 ng/mL	MS2/MS3	
D5-Diazepam	19	991	6.52	-0.16	289.88	0.12	7.5 E7		MS2/MS3	
Medazepam	8	912	1.86	0.12	270.84	0.16	2.9 E7	6 ng/mL	MS2/MS3	
Midazolam	6	990	1.74	0.08	325.89	0.21	2.5 E7	4 ng/mL	MS2/MS3	
Loprazolam	2	947	1.59	0.07	465.00	0.10	2.2 E7	6 ng/mL	MS2/MS3	
7-Aminoflunitrazepam	4	982	1.66	0.09	283.89	0.11	1.9 E7	5 ng/mL	MS2/MS3	
Metizolam	15	946	4.26	-0.05	328.90	0.10	1.7 E7	6 ng/mL	MS2/MS3	
Diclazepam	20	940	7.22	-0.05	319.03	-0.03	1.4 E7	5 ng/mL	MS2/MS3	
Clonazolam	10	981	3.07	0.23	353.92	0.18	1.2 E7	7 ng/mL	MS2/MS3	
Flunitrazepam	16	990	4.51	0.28	313.90	0.20	8.0 E6	5 ng/mL	MS2/MS3	
Alprazolam	13	964	4.03	-0.05	308.88	0.22	6.8 E6	5 ng/mL	MS2/MS3	
Clobazam	18	999	5.23	-0.08	300.89	0.11	5.5 E6	6 ng/mL	MS2/MS3	
Triazolam	14	991	4.25	-0.08	342.91	0.09	5.3 E6	6 ng/mL	MS2/MS3	
Flubromazepam	17	842	4.90	-0.07	332.84	0.16	2.8 E6	19 ng/mL	MS2/MS3	
Clonazepam	12	959	3.79	0.27	315.80	0.20	1.3 E6	< 5 ng/ml	MS2/MS3	
Chlordiazepoxide	3	822	1.62	0.11	299.89	0.11	7.4 E5	< 5 ng/ml	MS2/MS3	MS2 unspecific
Lorazepam	11	706	3.76	0.33	321.03	-0.03	6.0 E5	< 5 ng/ml	tentative	tentative

Figure 4: Results of semi-quant at 5 ng/mL.

Results and Discussion

The current spectral library contains 34 prescription benzodiazepines (most commonly prescribed in Germany), 21 designer benzodiazepines and three z-drugs, enabling screening for 58 benzodiazepine-type substances. The library can easily be extended with the emergence of new designer benzodiazepines entering 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, while 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 relevant trace concentrations in serum. Molecular ions of its 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. Semi-quantitative results in this preliminary study were found to vary within the range that fits the purpose of providing a good estimation of the concentration with deviations of ± 10 to ± 25% at medium concentrations. In contrast to MS/MS approaches, due to the data dependent acquisition of MS^n spectra (including the active exclusion of precursors) only MS^1 scan data is available for quantitation. For coeluting compounds, this leads to a higher influence of 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 screening data using single-point calibration. The Toxtyper software automatically processes full scan data from a routine screening approach, thus no modification to the acquisition method is required. Using customized calibration levels and suitable linear ranges, the accuracy obtained allows the discrimination of therapeutic, sub-therapeutic and potentially toxic serum levels.

As previously indicated, use of internal standards is essential for quantifying serum samples, and confirmatory analysis using a validated quantitative approach is mandatory in forensic case work.

Conclusions

The presented method allows automated identification and semi-quantitative determination of 58 benzodiazepines, including 19 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 research and forensic analysis. The automated semi-quantitative analysis enables a quick toxicological evaluation of the results and aids in the establishment of subsequent analytical strategies in cases of limited sample availability. Although this approach requires a more time consuming sample preparation when compared to routine immunoassays, unambiguous identification and semi-quantitative determination of compounds offers more detailed sample information.

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