Identification and characterization of the new designer benzodiazepines bromazolam, flualprazolam and fluclotizolam

Wilde, Maurice, Forensic Toxicology, Institute of Forensic Medicine, Medical Center – University of Freiburg, Freiburg, Germany
Sommer, Michaela, Forensic Toxicology, Institute of Forensic Medicine, Medical Center – University of Freiburg, Freiburg, Germany
Auwärter, Volker, Forensic Toxicology, Institute of Forensic Medicine, Medical Center – University of Freiburg, Freiburg, Germany

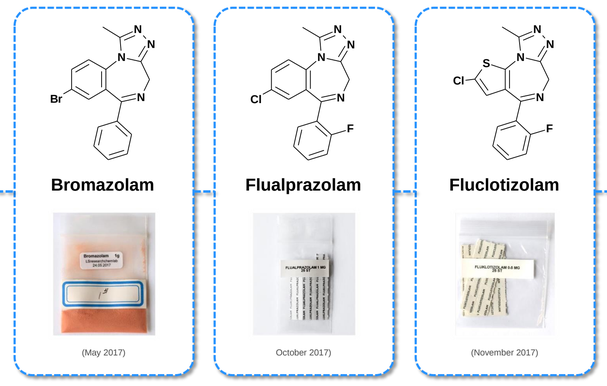
UNIVERSITÄTS KLINIKUM FREIBURG
IRM Institute of Forensic Medicine Forensic Toxicology

BACKGROUND & OBJECTIVES

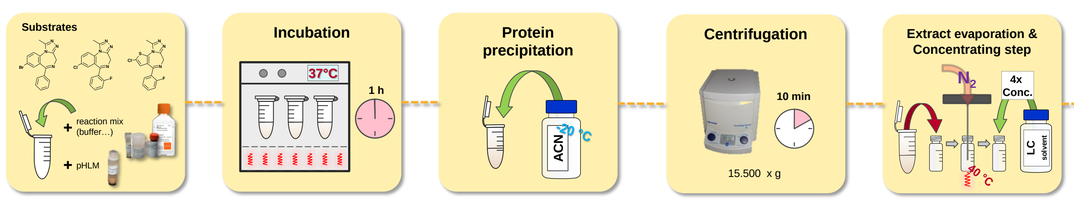
An increasing number of designer benzodiazepines (DBs) – a class of 'new psychoactive substances' (NPS) – was reported to the European monitoring centre for drugs and drug abuse (EMCDDA) in the last years. By the end of 2017 a total of twenty DBs have been reported to the EMCDDA. DBs have become a popular alternative for prescription drugs as self-medication or to counteract the effects and side effects of other drugs. Here we present the characterization of three new DBs that emerged on the European drug market in late 2016 and 2017. In addition, first *in vitro* metabolic studies were performed to identify phase I metabolites suitable as biomarkers for DB screening in human serum and urine samples.

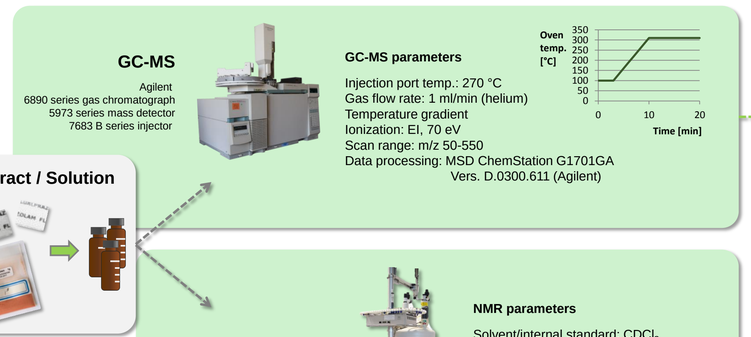
METHODS

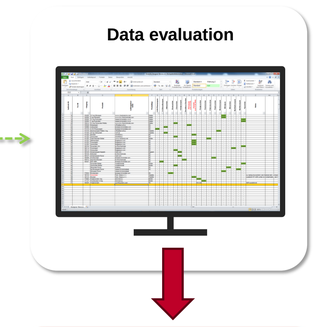
Online monitoring



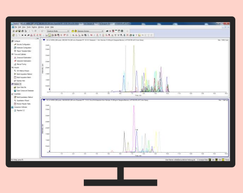
Studies of *in vitro* phase I metabolism using pooled human liver microsomes (pHLM)







Update of LC-MS/MS Screening for designer benzodiazepines



RESULTS

Structure identification and characterization

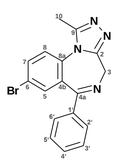
GC-EI-MS analysis

Flipchart GC-MS spectra

GC-EI-MS Spectra Library

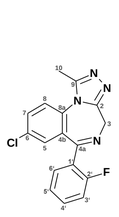
GC-EI-MS analysis confirmed identity of the purchased substances in all three products. Calculation of the peak areas using the integration algorithm of MSD Chemstation gave estimated GC-EI-MS purities of >99% for bromazolam and fluclotizolam, and of >89% for flualprazolam. MS-Spectral data was conclusive with a theoretical fragmentation pattern and included in an inhouse GC-EI-MS spectra library (later compared with GC-EI-MS spectral data of commercially available reference material).

NMR analysis



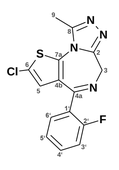
ID	# of protons	Chemical shift [ppm]	Multiplicity	Spin-spin coupling [Hz]
H3[a]	1	4.08	d	12.89
H3[b]	1	5.49	d	12.89
H5	1	7.59	d	2.23
H7	1	7.8	dd	2.23
				8.61
H8	1	7.35	d	8.66
H10	3	2.64	s	---
H2'+H6'	2 (1+1)	7.51-7.56	m	---
H3'+H5'	2 (1+1)	7.37-7.42	m	---
H4'	1	7.44-7.50	m	---

ID	Chemical shift [ppm]	Multiplicity	Spin-spin coupling [Hz]
C2	155.14	---	---
C3	46.42	---	---
C4a	168.01	---	---
C4b	130.96	---	---
C5	134.75	---	---
C6	121.06	---	---
C7	134.75	---	---
C8	124.92	---	---
C8a	132.83	---	---
C9	150.24	---	---
C10	12.44	---	---
C1'	138.63	---	---
C2'+C6'	129.42	---	---
C3'+C5'	128.58	---	---
C4'	131.07	---	---



ID	# of protons	Chemical shift [ppm]	Multiplicity	Spin-spin coupling [Hz]
H3[a]	2	4.12	d	12.95
H3[b]		5.54	d	12.95
H5	1	7.33	d	2.31
H7	1	7.63	dd	2.35
				8.67
H8	1	7.41	d	8.67
H10	3	2.64	s	---
H3'	1	7.02	ddd	0.98
				8.32
				9.33
H4'	1	7.43-7.5	m	
H5'	1	7.25	dt	1.12
				7.59
H6'	1	7.66	dt	1.84
				7.65

ID	Chemical shift [ppm]	Multiplicity	Spin-spin coupling [Hz]
C2	154.89	---	---
C3	46.5	---	---
C4a	165.17	---	---
C4b	131.01	---	---
C5	130.17	---	1.53
C6	133.82	---	---
C7	131.79	---	---
C8	124.65	---	---
C8a	131.9	---	1.54
C9	150.57	---	---
C10	12.4	---	---
C1'	127.19	d	11.45
C2'	160.39	d	254
C3'	116.42	d	21.8
C4'	132.81	d	8.43
C5'	124.79	d	3.84
C6'	131.2	d	1.93



ID	# of protons	Chemical shift [ppm]	Multiplicity	Spin-spin coupling [Hz]
H3	2	4.94	s (broad)	---
H5	1	6.73	d	1.26
H9	3	2.71	s	---
H3'	1	7.08	ddd	0.99
				8.33
				9.35
H4'	1	7.46	dddd	1.8
				5.13
				7.28
				8.27
H5'	1	7.23	dt	1.01
				7.56
H6'	1	7.56	dt	1.79
				7.61

ID	Chemical shift [ppm]	Multiplicity	Spin-spin coupling [Hz]
C2	153.41	---	---
C3	47.16	---	---
C4a	161.78	---	---
C4b	126.94	---	---
C5	125.78	d	2.23
C6	132.9	---	---
C7a	129.81	---	---
C8	149.56	---	---
C9	12.14	---	---
C1'	126.58	d	11.55
C2'	160.43	d	251.74
C3'	116.44	d	21.35
C4'	132.74	d	8.38
C5'	124.74	d	3.79
C6'	131.25	d	2.28

Flipchart NMR analysis

^{1}H and ^{13}C spectral data from NMR analysis was manually evaluated using TopSpin software (v. 3.5). With the help of 2D correlations (^{1}H-COSY, ^{1}H-^{13}C-HSQC and ^{1}H-^{13}C-HMBC experiments) all proton and carbon signals were unambiguously assigned to the chemical structures of the respective compound (data below).

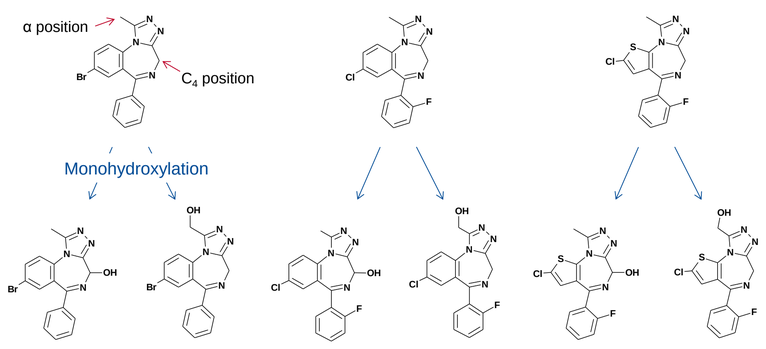
LC-qToF-MS data

Flipchart HLM data

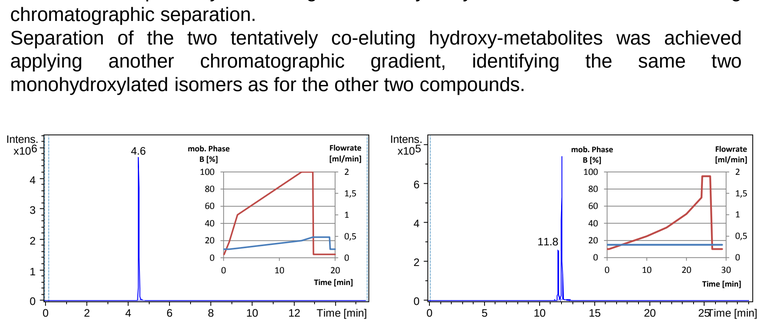
Identification of *in vitro* phase I metabolites

Only few phase I metabolites were detected for the investigated compounds in the *in vitro* microsomes' assay.
The only metabolic reaction observed assay was hydroxylation, resulting in mono-hydroxylated and dihydroxylated metabolites for all three benzodiazepines.

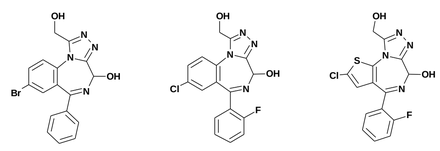
Parent compounds



For bromazolam and flualprazolam two monohydroxylated metabolites were identified, either hydroxylated at C_4 of the diazepine core or at the α-C atom of the triazolo ring. For fluclotizolam only one peak representing monohydroxylation was found at first, probably consisting of both hydroxylated isomers due to missing chromatographic separation.
Separation of the two tentatively co-eluting hydroxy-metabolites was achieved applying another chromatographic gradient, identifying the same two monohydroxylated isomers as for the other two compounds.



Dihydroxylation



One dihydroxylated metabolite was detected for all three compounds in full scan MS (most likely hydroxylated at the same positions), however no matching bbCID spectra were obtained for proper identification, due to low abundances of the signals.

CONCLUSIONS

The pHLM assay showed a distinct metabolic profile for the three compounds matching the metabolic pattern found for other triazolo benzodiazepines so far. Most tentatively identified *in vivo* phase I metabolites (monohydroxylated metabolites) were detected with relatively high abundances, suggesting these compounds as potential long term biomarkers.

Literature

[1] European database on new drugs (EDND) provided by the EMCDDA https://ednd.emcdda.europa.eu/html.cfm/index7246EN.html

Contact

Maurice Wilde
Medical Center – University of Freiburg
Institute of Forensic Medicine
Albertstraße 9
79104 Freiburg, Germany
maurice.wilde@uniklinik-freiburg.de