Method development for the analysis of substances relevant to § 24a (2) of the German road traffic act using LC-MS^n

Franziska Ehrhardt[1,2], Volker Auwärter[1], Jürgen Kempf[1]

[1]*Offenburg University of Applied Sciences, Offenburg, Germany*
[2]*Institute of Forensic Medicine, Forensic Toxicology, Medical Center - University of Freiburg, Germany*

UNIVERSITÄTS KLINIKUM FREIBURG
Institute of Forensic Medicine
Forensic Toxicology

Introduction

Numerous driving under the influence of drugs (DUID) cases in Germany are dealing with the question of suspected violation of § 24a (2) of the German road traffic act (GRTA). This *per se* regulation assumes driving under the influence of drugs (DUID) - and therefore a traffic offense - if serum concentrations of amphetamine, methamphetamine, 'ecstasy' (MDMA, MDA or MDE), morphine, cocaine (or benzoylecgonine (BE)), or THC exceed certain levels. In the lab, serum samples are usually pre-screened by immunoassay (IA) and positive results are confirmed by LC-MS/MS or GC-MS since neither the qualitative nor the quantitative information from immunoassays is admissible in court. Antibody based tests may lead to false positive results due to cross reactivity issues caused by various other compounds or matrix components or even false negative results due to low sensitivity. This increases the workload for confirmation analyses, usage of sample volume, and the overall costs. However, the great benefits of IAs are the high degree of automation regarding sample preparation and reporting of results and their overall ease-of-use.

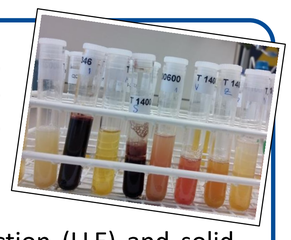
The aim of this work was to develop a fast and automated LC-MS^n method for the detection of this compounds, combining the ease-of-use of an IA with unambiguous compound identification of MS analysis.

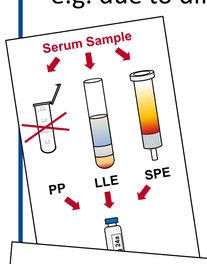
Cut-offs §24a (2) GRTA

Compound	Serum Conc.
Morphine, Cocaine	10 ng/mL
Amphetamine	25 ng/mL
Methamphetmaine	25 ng/mL
MDMA, MDA, MDEA	25 ng/mL
Benzoylecgonine (BE)	75 ng/mL
THC	1.0 ng/mL

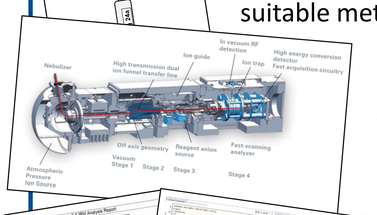
Experimental

The need for sample preparation is a drawback of MS analysis. Besides the high selectivity and sensitivity of today MS, finding an appropriate sample preparation is crucial for analysis of serum samples, which often differ in matrix load e.g. due to different states of hemolysis, lipid content etc.



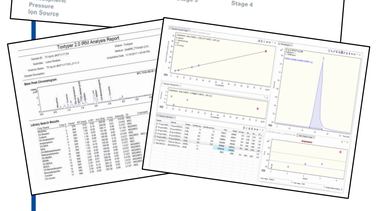


Protein precipitation (PP), liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are the most common extraction methods in forensic toxicology. PP was excluded at the very beginning of this project due to insufficient sensitivity. Two in-house used SPE and two LLE methods were tested in more detail and LLE of 500 µL serum using chloroform/ isopropanol was found to be the most suitable method.



In cooperation with the application team of Bruker Daltonik, the parameters of the ion transfer of the MS were optimized to reduce in-source fragmentation and loss of small molecules before entering the ion trap.

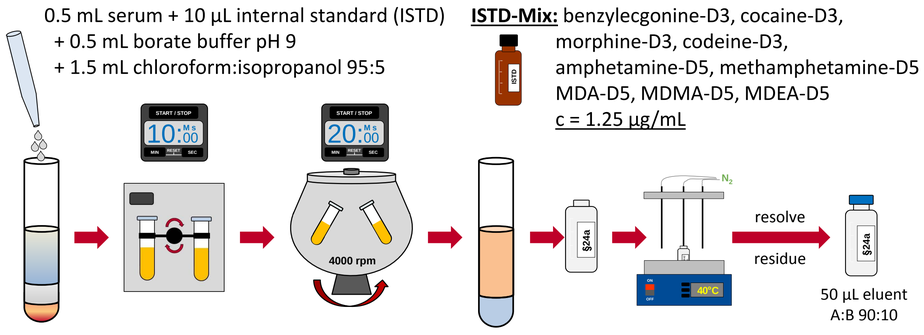
Two MS^n modes were evaluated: AutoMSn mode for automated detection, identification by library search and automated reporting (Toxtyper workflow[1]) and smartMRM mode using data independent acquisition (DIA) of MS² data for identification and quantitation - both using a scheduled precursor list (SPL).



Sample Preparation

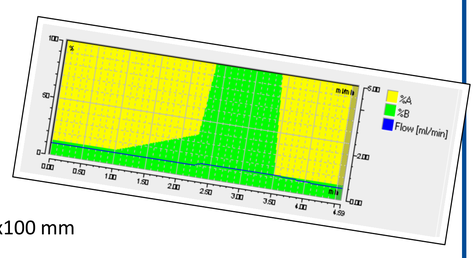
0.5 mL serum + 10 µL internal standard (ISTD)
+ 0.5 mL borate buffer pH 9
+ 1.5 mL chloroform:isopropanol 95:5

ISTD-Mix: benzylecgonine-D3, cocaine-D3, morphine-D3, codeine-D3, amphetamine-D5, methamphetamine-D5 MDA-D5, MDMA-D5, MDEA-D5
c = 1.25 µg/mL



LC - MS^n 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
Gradient: 4.5 min gradient elution
Column: Acclaim® RSLC 120 C18 2,2 µm 120A 2.1x100 mm



MS-System: Bruker amaZon speed™ ion trap
Ion source: ESI source, positive mode, Capillary: 2500 V, Dry Temp.: 160 °C
Scan mode: UltraScan (70 - 400 Da at 32.500 Da/s)
MSn mode AutoMSn (DDA) / smartMRM (DIA)
SPL: SPL for AutoMSn and smart MRM
Ion transfer:

Cap Exit			RF Level		
80.0			30 %		
	Funnel 1	Funnel 2	Octopole	DC1	DC2
In	60	12		1.6	0.6
Out	35	4.5	Multipole	-6.0	
Lense	25	4.0			

Validation Parameters

Both methods were validated according to the guidelines of the German Society of Toxicological and Forensic Chemistry (GTFCh)[2] for quantitative LC-MS methods.

Selectivity: Blank serum samples of 10 individuals, two serum samples fortified with 9 internal standards (ISTD), and serum samples fortified with methadone/EDDP, common benzodiazepines and psychotropic medical drugs were analyzed to evaluate selectivity of both methods.

LOD: LODs were determined using calibrators with decreasing concentrations around the requested cut-off concentrations. LOD was defined as the concentration that could be identified automatically in three-fold determination (AutoMSn) or the concentration with a S/N ratio greater than 3 (smartMRM).

LOQ (smartMRM): LOQ was defined as the concentration with a S/N ratio greater than 10.

Linearity (smartMRM): For determination of linearity, six calibration curves were analyzed. Each calibration consisted of six calibrators, made by fortifying blank pooled serum (n = 5) with a mixture of all target analytes in acetonitrile.

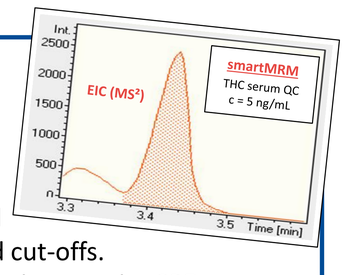
Accuracy (smartMRM): Two replicates of a low, medium and high QC sample (10, 25 and 75 ng/mL) were analyzed on eight consecutive days.

Matrix effects (smartMRM): Matrix effects (ME) were examined according to Matuszewski et al.[3] using a low and high QC sample.

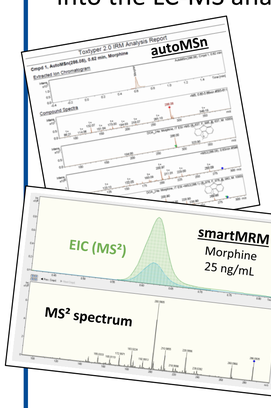
Stability (smartMRM): To evaluate stability of the samples in the autosampler, six aliquots of a high and low QC sample were analyzed every 4 hours during a 24 h time period.

Results

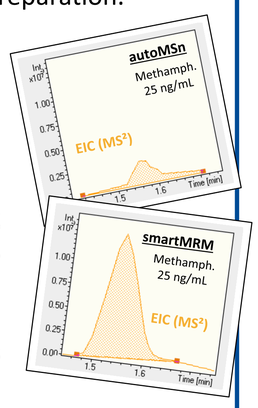
Unfortunately, neither the SPE nor the LLE methods tested for sample preparation allowed extraction of all the alkaline drugs **and** THC. Extraction efficiency of THC, and therefore signal intensity, was insufficient to detect the requested cut-off concentration. So THC was excluded from further method development.



Both SPE methods - routinely used for quantitative analysis of alkaline drugs[4] and general unknown screening[5] in the lab - enabled detection of all compounds below the requested cut-offs. However, due to the high cost of SPE cartridges and the missing opportunities to fully implement the SPE process into the LC-MS analysis at this time, an easy but sufficient LLE procedure was chosen for sample preparation.



The easiest and most efficient way to distinguish positive from negative samples is screening the samples using AutoMSn mode with fully automated data evaluation and reporting. Due to data dependent acquisition of spectra - including dynamic exclusion - there is only a limited number of data point in MS² that can be used for quantitation.



To gain quantitative information, the smartMRM mode - acquiring information independent MS² data - was evaluated. Identification is performed either by library matching of MS² spectra or calculation of ion ratios of EIC traces from MS² data similar to common MRM approaches. The latter can also be used to gain quantitative results. Unfortunately, this approach is not yet fully automatable.

Validation Results

Selectivity: Blank serum samples, blank serum samples fortified with ISTDs and/or benzodiazepines, psychotropic medical drugs and methadone led to no automated positive findings of target analytes in the reports of the AutoMSn mode. Single tentative findings could easily be ruled out by inspection of the applied library matches. These samples also showed no interfering signals on the ion transitions of the analytes in smartMRM mode.

LOD (AutoMSn): LODs were found to be 2.5 ng/mL for amphetamine, methamphetamine, MDA, MDEA and morphine. BE, codeine and MDMA could be identified automatically at 5.0 ng/mL and cocaine at 7.5 ng/mL in three-fold determination.

LOD/LOQ (smartMRM): The lowest tested serum calibration point c = 2.5 ng/mL showed signal-to-noise ratios greater than 20 for all of the target analytes. So LOD and LOQ of the smartMRM approach was set to 2.5 ng/mL.

Linearity: Linearity was evaluated using 6 six-point calibration curves (7.5 - 100 ng/mL). The average of the coefficients of determination (R^2) ranged from 0.9916 to 0.9969 with relative standard deviations (rel. SD) of 1% or lower.

Accuracy: Accuracy was calculated as bias for all three QC levels. For QC_{Low} (c = 10 ng/mL) it was found to be less than ± 20% except for BE. For QC_{Med} (c = 25 ng/mL) and QC_{High} (c = 75 ng/mL) requested deviations of ± 15% for quantitative LC-MS analysis could not be met for all compounds. Especially cocaine and BE clearly exceeded that range. Repeatability was below 20% for QC_{LOW} and below 10% for QC_{Med} and $QC_{High,}$ respectively. The high bias of cocaine and BE was supposed to derive from degradation of cocaine during the freeze-thaw cycle of the QC samples.

Matrix effects: ME for QC_{Low} ranged from 86 to 123% (SD: 5 to 44%) and from 97 to 118% (SD: 10 to 38%) for QC_{High}.

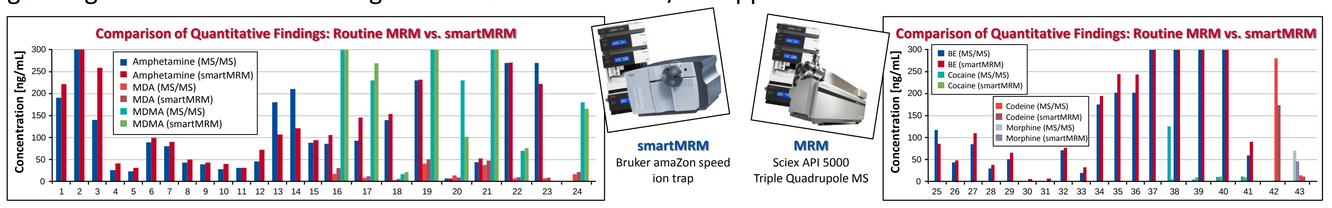
Stability: According to the guidelines of the GTFCh, Peak areas should not decrease more than 25% during the runtime of a batch. For QC_{Low} samples this criteria was met for all compounds. Peak area of QC_{High} samples in general showed higher variations than for QC_{Low}. After 20 h, MDA, MDMA and MDEA showed signal loss between 30 and 40%.

Analysis of Real Serum Samples

60 randomly selected case samples sent in for suspected violation of § 24a (2) GRTA were reanalyzed using both LC-MS^n methods. Results were compared with the original findings from routine IA and LC-MS/MS (MRM) analysis.

AutoMSn: Automated findings of the AutoMSn method corresponded with the LC-MS/MS findings if concentrations were above the evaluated LODs. False positive IA findings (opiates, methamphetamine) were found to be negative.

SmartMRM: Except two false positive cases (BE 5.0 ng/mL), qualitative results from the smartMRM method were in good agreement with the findings from the routine LC-MS/MS approach.



Since the upper limit of quantitation of both methods is below 300 ng/mL (MRM: 250 ng/mL, smartMRM: 100 ng/mL), the y-axis were limited to 300 ng/mL for better graphical representation. Due to legal regulations, only samples older than two years could be used for evaluation of the methods. This storage time may explain some of the significantly lower concentrations determined by smart MRM (e.g. # 13, 14, 21, 38, 42).

Nevertheless, considering the analytical question of suspected violation of § 24a (2) GRTA, all results found below/above the respective legal cut-off in routine casework could be reproduced using smartMRM except for one case. In case #5, amphetamine levels determined by smartMRM (c = 31 ng/mL) were above the legal cut-off, while a concentration of 23 ng/mL was quantified by routine LC-MS/MS. Since every positive LC-MS^n result - autoMSn or smartMRM - would be confirmed by quantitative LC-MS/MS, this is discrepancy is negligible for routine casework.

Conclusion

Both LC-MS^n modes enable fast and reliable detection and identification of drugs relevant to § 24a (2) GRTA (except THC) below their respective cut-off concentrations, making them a suitable tool for screening serum samples in suspected DUID cases. Although accuracy requirements were not met for all compounds, quantitative information can still be used for a quick assessment of the case or to decide on appropriate dilution for subsequent LC-MS/MS analysis. Regarding the short runtime and the daily sample load, intensity loss of the designer amphetamines after 20 h in the autosampler is not regarded as an issue in everyday routine work.

The analytical results of 60 random DUID cases could be confirmed by the two screening methods, except for two false-positive BE findings in smartMRM mode. No false negative results occurred.

Although sample preparation is still carried out manually at this point, the developed LC-MS^n approach would be a suitable replacement for IA testing in DUID cases according to § 24a (2) GRTA.

The next step of this project is the implementation of an online sample preparation and the development of scripts for automated evaluation of smartMRM data to fully automate the complete process similar to IA screening.

Acknowledgements

Consumables for this bachelor thesis were funded by the "Bund gegen Alkohol und Drogen im Straßenverkehr" (B.A.D.S.).

Literature

[1] *Kempf et. al.: Forensic Sci Int. 243: 84-9*
[2] *www.gtfch.org/cms/index.php/en/guidelines*
[3] *Matuszewski et al.: Anal. Chem. 75: 3019-3030*
[4] *Weinmann et al.: Int J Legal Med 113: 229-235*
[5] *Pelander et al.: JAT 34(6): 312-8*