Using LC-QTOF-MS in validated screening workflows in forensic toxicology: A qualitative/quantitative method for 93 drugs of abuse in human urine samples

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Background

Full scan based screening methods using LC-QTOF-MS are a valuable tool for forensic analysis due to the possibility of qualitative/quantitative and retrospective data evaluation in a single run. In this study a previously developed LC-QTOF-MS screening workflow was validated for qualitative and quantitative analysis of drugs and drugs of abuse in human urine.

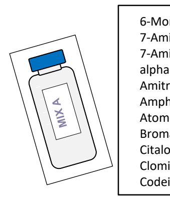
Objective

A basic validation including limits of detection, limits of quantitation, linearity, accuracy, selectivity, and precision was carried out. Furthermore, the methods' limitations regarding its applicability to urine screening in post-mortem toxicology, workplace drug testing, drug-facilitated crime (DFC), and intoxication cases were evaluated. Special focus was given to prove that cut-off values for sobriety and fitness-to-drive testing are met.

Methods I

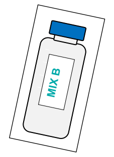
Compounds of Interest

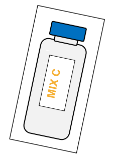
For this evaluation 93 of the most common drugs and drugs of abuse and their metabolites detected in routine case work of our institute were chosen.



6-Monoacetylmorphine (MAM)
7-Aminoclonazepam
7-Aminoflunitrazepam
alpha-Hydroxymidazolam
Amitriptyline
Amphetamine
Atomoxetine
Bromazepam
Citalopram
Clomipramine
Codeine
Desmethyldoxepin
Doxepin
Doxylamine
Ecgonine methyl ester
Flubromazepam
Gabapentin
Ketamine
MDEA
Midazolam
Mirtazapine
Norclomipramine
Nordazepam
O-Desmethylvenlafaxin
Opipramol
Paracetamol (Acetaminophen)
Pregabaline
Quetiapin
Remifentanyl
Risperidon
Triazolam
Zolpidem

Carbamazepine
Clonazepam
Cocaethylen
Desipramine
Diphenhydramine
Fentanyl
Flunitrazepam
Haloperidol
Levetiracetam
MDA
MDMA
Melperone
Methadone
Methylphenidate
Morphine
Norfentanyl
Nortilidine
Nortriptylin
Oxazepam
Oxycodone
Piritramide
Promazine
Ritalinic acid
Temazepam
Tilidine
Trazodone
Trimipramine
Venlafaxine
Etizolam





9-Hydroxyrisperidone (Paliperidone)
Alprazolam
Amisulpride
Benzoylecgonine
Buprenorphine
Carbamazepine-epoxide
Clozapine
Cocaine
Diazepam
Dihydrocodeine
EDDP
Fluoxetine
Lamotrigine
Lorazepam
mCPP
Methamphetamine
Metoclopramide
Norbuprenorphine
Norcitalopram
Noroxycodone
Nortrimipramine (Imipramine)
O-Desmethyltramadol
Olanzapine
Paroxetin
Pethidine
Promethazine
Sertraline
Sufentanil
THC-COOH
Tramadol
Zopiclone

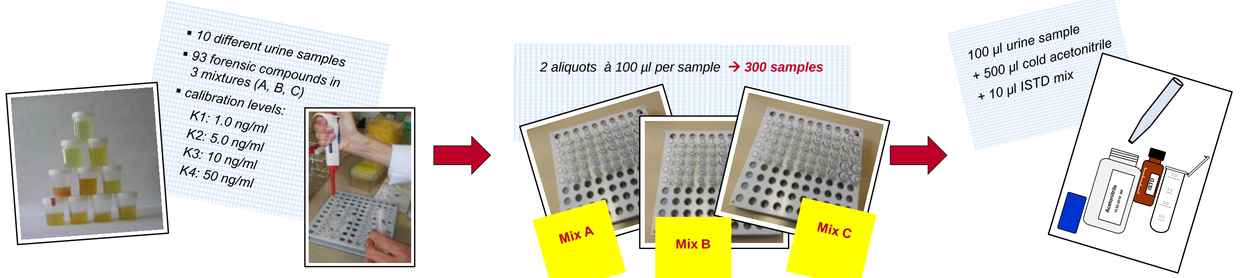
Disclosure:

The presenting author has no financial relationships with a company as defined in the AACC policy on disclosure of potential bias or conflict of interest.

Methods II

Sample Preparation

Ninety three substances of forensic relevance were spiked into ten different urine samples at the concentrations 1.0, 5.0, 10, and 50 ng/ml.

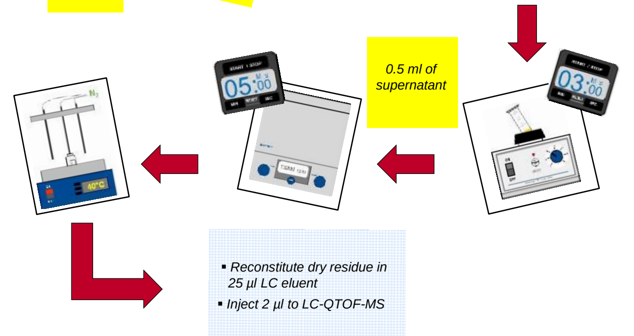


- 10 different urine samples
- 93 forensic compounds in 3 mixtures (A, B, C)
- calibration levels:
  K1: 1.0 ng/ml
  K2: 5.0 ng/ml
  K3: 10 ng/ml
  K4: 50 ng/ml

2 aliquots à 100 µl per sample → 300 samples

100 µl urine sample
+ 500 µl cold acetonitrile
+ 10 µl ISTD mix

Samples were precipitated with cold acetonitrile after addition of seven isotope labeled compounds as internal standards (ISTD).
The dried residues were reconstituted and analyzed in duplicate on two LC-QTOF-MS systems in two different labs.



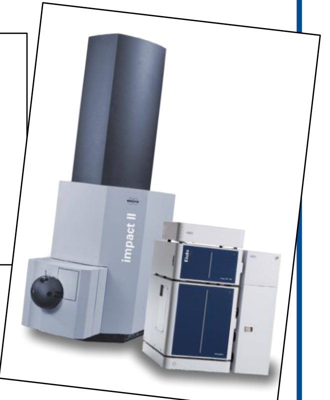
- Reconstitute dry residue in 25 µl LC eluent
- Inject 2 µl to LC-QTOF-MS

Analytical Methods

Separation was performed on a Bruker Intensity Solo C18 column using a 14 min gradient elution. The MS (Bruker impact II) was operated in positive electrospray ionization mode generating a full scan and broad band CID spectra (bbCID) using collision energy spread (24 - 36 eV).

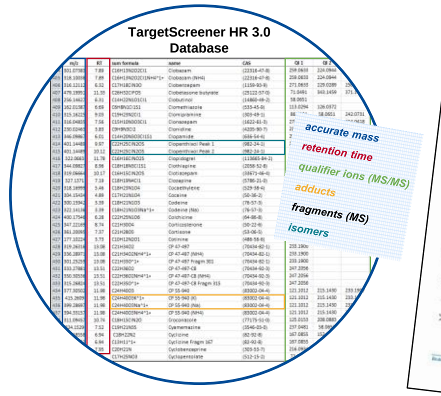
UHPLC: Bruker Elute UHPLC
Column: Bruker Intensity Solo 1.8 C18-2, 2.1*100 mm and pre-column
Mobile phase A: H2O/MeOH 99/1, 5 mM NH4 formate / 0.01% HCOOH
Mobile phase B: MeOH, 5 mM NH4 formate / 0.01% HCOOH
Gradient: multistep gradient 5 - 99.9% in 15 min (20 min cycle)
Flow rate: flow gradient 0.2 - 0.48 ml/min,
Injection vol.: 2 µl
Column temp.: 40°C

MS: Bruker impact II QTOF mass spectrometer
Ionization: ESI(+), 2,500V
Scan range: m/z 30 - 1000
Full scan rapidly alternating TOF MS (4eV) with bbCID (30eV +/- 6V) @ 2Hz



Data Analysis

Data evaluation was performed with TASQ 1.4 software using the TargetScreener HR 3.0 accurate masse database containing mass spectrometric and chromatographic information of 2184 drugs, drugs of abuse, new psychoactive substances (NPS) metabolites, and pesticides.



TargetScreener HR 3.0 Database

accurate mass
retention time
qualifier ions (MS/MS)
adducts
fragments (MS)
isomers

Results

Limits of Detection (LOD) and Selectivity

LOD was set to the concentration at which a substance was detected in 95% of all measurements (n = 40, due to duplicate determination) according to the identification criteria on the right.

Identification Criteria

- *retention time ± 0.3 min*
- *signal to noise ratio > 3:1 for all ions*
- *$[M+nH]^{n+}$ and $[M+nH+1]^{n+}$ detected (MS)*
- *at least two qualifier ions with minimum one being a true fragment of the molecular ion (bbCID)*

Identification at the lowest concentration (c = 1.0 ng/ml) was achieved for 60 % of the tested compounds. Only five compounds (paracetamol, THC-COOH, norclomipramine, piritramid, and levetiracetam) could not be detected in all samples at the investigated concentrations. This is probably due to matrix effects and/or low ionization yields.

Except THC-COOH and ethylglucuronide, most substances with legal cut-offs according to German regulations for abstinence screening in fitness-to-drive assessment (CTU3 criteria), were detected well below the respective requested cut-off concentrations.

Typical 'date rape drugs' like flunitrazepam, doxylamine, and diphenhydramine showed LODs sufficient for detection of a recent uptake of these drugs.

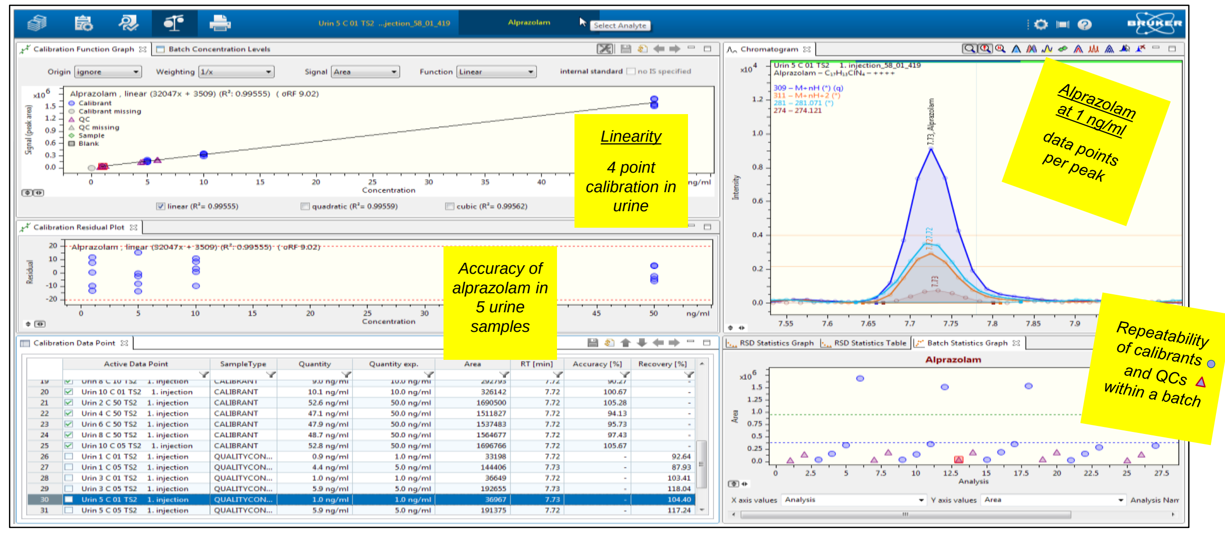
Designer benzodiazepines and fentanyl derivatives were detected with extraordinarily high sensitivity.

Minimum LOD requirements according to German CTU3 Criteria

Compounds	Cut-off (CTU3)	LC-QTOF LOD
Amphetamines:		
Amphetamine	50 ng/ml	5.0 ng/ml
MDA	50 ng/ml	10 ng/ml
MDEA	50 ng/ml	10 ng/ml
MDMA	50 ng/ml	10 ng/ml
Methamphetamine	50 ng/ml	50 ng/ml
Cocaine:		
Benzoylecgonine	30 ng/ml	10 ng/ml
Cocaine	30 ng/ml	5.0 ng/ml
Opiates:		
Codeine	25 ng/ml	10 ng/ml
Dihydrocodeine	25 ng/ml	5.0 ng/ml
Morphine	25 ng/ml	5.0 ng/ml
Methadone:		
EDDP	50 ng/ml	1.0 ng/ml
Methadone	50 ng/ml	1.0 ng/ml
Benzodiazepines:		
Diazepam	50 ng/ml	1.0 ng/ml
Nordazepam	50 ng/ml	1.0 ng/ml
Oxazepam	50 ng/ml	1.0 ng/ml
Alprazolam	50 ng/ml	1.0 ng/ml
Hydroxyalprazolam	50 ng/ml	not investigated
Bromazepam	50 ng/ml	1.0 ng/ml
Flunitrazepam	50 ng/ml	1.0 ng/ml
7-Aminoflunitrazepam	50 ng/ml	50 ng/ml
Lorazepam	50 ng/ml	1.0 ng/ml
Cannabinoids:		
THC-COOH	10 ng/ml	> 50 ng/ml
Alcohol:		
Ethylglucuronide	100 ng/ml	not investigated

Quantitative Results

The linear dynamic ranges were four magnitudes or greater. LOQ was set to the lowest LOD.
The precision ranged from 8 % to 30 %. Overall accuracy met the criteria for bioanalytical method validation according to forensic guidelines.
The method showed good selectivity/specificity fulfilling the requirements stated in the respective guidelines.



Urin 5 C 01 TS2 ...jection_58_01_419 · Alprazolam · Select Analyte

Calibration Function Graph · Batch Concentration Levels

Origin: ignore · Weighting: 1/x · Signal: Area · Function: Linear · internal standard ☐ no IS specified

Alprazolam , linear (32047x + 3509) (R²: 0.99555) (σRF 9.02)

☑ linear (R²= 0.99555) ☐ quadratic (R²= 0.99559) ☐ cubic (R²= 0.99562)

Calibration Residual Plot

Alprazolam , linear (32047x + 3509) (R²: 0.99555) (σRF 9.02)

Chromatogram

Urin 5 C 01 TS2 1. injection_58_01_419
Alprazolam – C17H13ClN4 – ++++
309 – M+nH (*) (q)
311 – M+nH+2 (*)
281 – 281.071 (*)
274 – 274.121

Calibration Data Point

		Active Data Point		SampleType	Quantity	Quantity exp.	Area	RT [min]	Accuracy [%]	Recovery [%]
20	☑	Urin 10 C 01 TS2	1. injection	CALIBRANT	10.1 ng/ml	10.0 ng/ml	326142	7.72	100.67	-
21	☑	Urin 2 C 50 TS2	1. injection	CALIBRANT	52.6 ng/ml	50.0 ng/ml	1690500	7.72	105.28	-
22	☑	Urin 4 C 50 TS2	1. injection	CALIBRANT	47.1 ng/ml	50.0 ng/ml	1511827	7.72	94.13	-
23	☑	Urin 6 C 50 TS2	1. injection	CALIBRANT	47.9 ng/ml	50.0 ng/ml	1537483	7.72	95.73	-
24	☑	Urin 8 C 50 TS2	1. injection	CALIBRANT	48.7 ng/ml	50.0 ng/ml	1564677	7.72	97.43	-
25	☑	Urin 10 C 05 TS2	1. injection	CALIBRANT	52.8 ng/ml	50.0 ng/ml	1696766	7.72	105.67	-
26	☐	Urin 1 C 01 TS2	1. injection	QUALITYCON...	0.9 ng/ml	1.0 ng/ml	33198	7.72	-	92.64
27	☐	Urin 1 C 05 TS2	1. injection	QUALITYCON...	4.4 ng/ml	5.0 ng/ml	144406	7.73	-	87.93
28	☐	Urin 3 C 01 TS2	1. injection	QUALITYCON...	1.0 ng/ml	1.0 ng/ml	36649	7.72	-	103.41
29	☐	Urin 3 C 05 TS2	1. injection	QUALITYCON...	5.9 ng/ml	5.0 ng/ml	192655	7.73	-	118.04
30	☐	Urin 5 C 01 TS2	1. injection	QUALITYCON...	1.0 ng/ml	1.0 ng/ml	36967	7.73	-	104.40
31	☐	Urin 5 C 05 TS2	1. injection	QUALITYCON...	5.9 ng/ml	5.0 ng/ml	191375	7.72	-	117.24

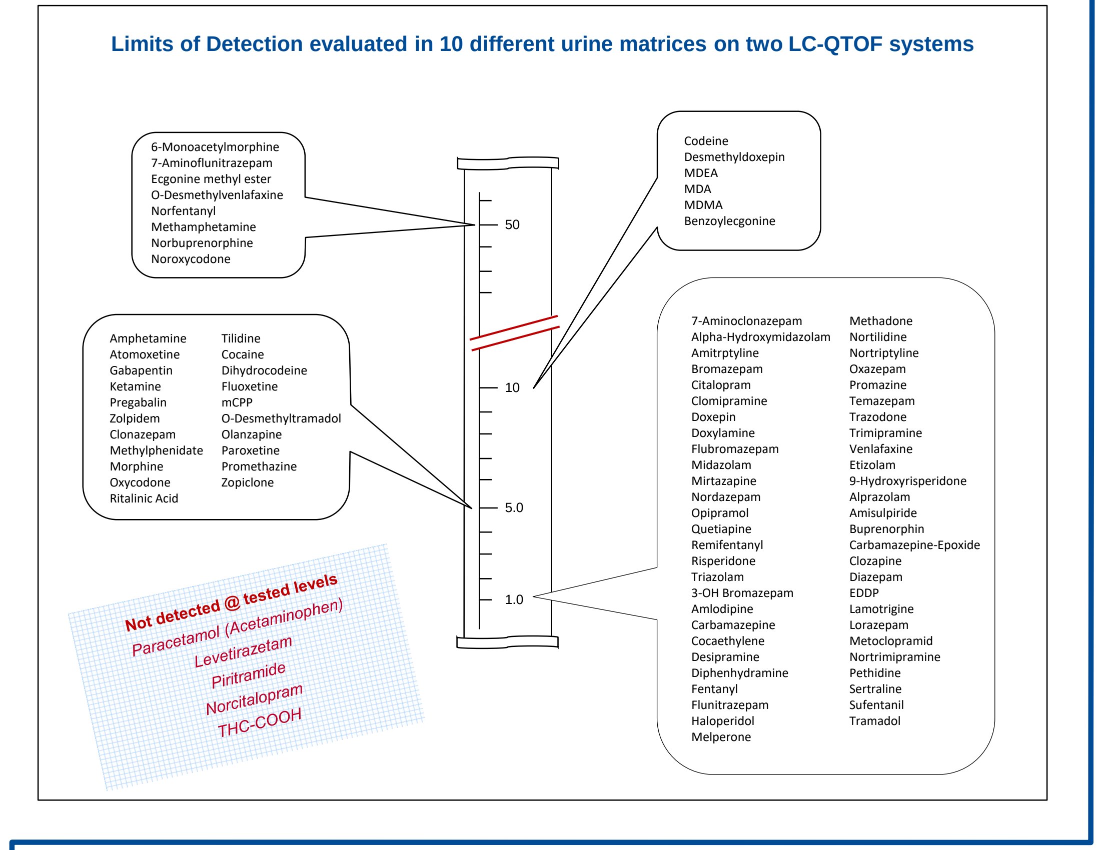
RSD Statistics Graph · RSD Statistics Table · Batch Statistics Graph

Linearity
4 point calibration in urine

Accuracy of alprazolam in 5 urine samples

Alprazolam at 1 ng/ml
data points per peak

Repeatability of calibrants ○ and QCs △ within a batch



Limits of Detection evaluated in 10 different urine matrices on two LC-QTOF systems

6-Monoacetylmorphine
7-Aminoflunitrazepam
Ecgonine methyl ester
O-Desmethylvenlafaxine
Norfentanyl
Methamphetamine
Norbuprenorphine
Noroxycodone

Codeine
Desmethyldoxepin
MDEA
MDA
MDMA
Benzoylecgonine

Amphetamine
Atomoxetine
Gabapentin
Ketamine
Pregabalin
Zolpidem
Clonazepam
Methylphenidate
Morphine
Oxycodone
Ritalinic Acid
Tilidine
Cocaine
Dihydrocodeine
Fluoxetine
mCPP
O-Desmethyltramadol
Olanzapine
Paroxetine
Promethazine
Zopiclone

7-Aminoclonazepam
Alpha-Hydroxymidazolam
Amitrptyline
Bromazepam
Citalopram
Clomipramine
Doxepin
Doxylamine
Flubromazepam
Midazolam
Mirtazapine
Nordazepam
Opipramol
Quetiapine
Remifentanyl
Risperidone
Triazolam
3-OH Bromazepam
Amlodipine
Carbamazepine
Cocaethylene
Desipramine
Diphenhydramine
Fentanyl
Flunitrazepam
Haloperidol
Melperone
Methadone
Nortilidine
Nortriptyline
Oxazepam
Promazine
Temazepam
Trazodone
Trimipramine
Venlafaxine
Etizolam
9-Hydroxyrisperidone
Alprazolam
Amisulpiride
Buprenorphin
Carbamazepine-Epoxide
Clozapine
Diazepam
EDDP
Lamotrigine
Lorazepam
Metoclopramid
Nortrimipramine
Pethidine
Sertraline
Sufentanil
Tramadol

Not detected @ tested levels
Paracetamol (Acetaminophen)
Levetirazetam
Piritramide
Norcitalopram
THC-COOH

Conclusion

In this project, the analytical possibilities and limitations of an LC-QTOF approach for screening urine samples were evaluated using 93 forensic compounds with high prevalence in our everyday case work.

For a screening method, selectivity and LODs are the most important analytical parameters. Evaluated LODs were comparable with those of standard triple quadrupole (QqQ) methods for the majority of compounds investigated. Although, high end QqQ may reach lower LODs, considering the high number of analytes due to full scan analysis, LC-QTOF is a valuable tool for toxicological analysis and the presented LODs are sufficient for most analytical problems in everyday case work.

Given the high frequency of new psychoactive substances emerging on web-based drug markets and related fatalities, this is of particular interest to the forensic field due to the possibility of retrospective data evaluation.

Extrapolating the here presented urine analysis results, application to blood and hair samples seems promising and will be evaluated in a subsequent study.

Acknowledgement

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