APPNOTE

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GERSTEL AppNote 236

Determining Psychoactive Drugs in Blood Plasma and Serum Using Automated SPE–LC–MS/MS

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Keywords

Blood Serum, Benzodiazepines, Sedatives, Antidepressants, Antipsychotics, Methadone, Metabolites, Solid Phase Extraction (SPE), Automation, LC-MS/MS

Abstract

Determination of psychoactive drugs in the context of clinical and forensic toxicology, down to the low ng/mL range from only 250 µL of blood plasma or serum, is described in this work. A comprehensively automated "multi method" based on solid-phase extraction (SPE) coupled online to LC–MS/MS is used to determine 65 compounds, including benzodiazepines, various sedatives, antidepressants, antipsychotics, methadone, and their relevant metabolites. The method has been fully validated for 52 of those compounds following the guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh). Semi-quantitative or qualitative determination of some less relevant drugs and metabolites is also included in this work.

Introduction

Psychoactive drugs play a major role in clinical or forensic cases investigated by toxicology laboratories. In addition to typical illicit drugs, such as, for example, amphetamines, opioids, and cannabinoids, so-called new psychoactive substances (NPS) are often sold via the internet as legal highs, bath salts, or research chemicals. This trend has emerged in the last decade.

The determination of illicit drugs and prescription drugs in blood plasma or serum and other biological matrices, is the main focus of this work. Benzodiazepines, which are predominantly prescribed as sedatives, anxiolytics, and hypnotics are the most widely used therapeutic drugs in psychiatry. Common for all benzodiazepines is the bicyclic structure consisting of a condensed diazepine- and benzene ring. Some well-known active pharmaceutical ingredients (API) are diazepam, lorazepam, and midazolam. Although benzodiazepines are less toxic than their predecessors, the barbiturates, intoxications can occur and can be fatal, especially in combination with other drugs such as alcohol and opioids, and long-term use can lead to physical addiction.

Sedatives that act in a similar way to benzodiazepines, but have a different chemical structure, are referred to as non-benzodiazepines or sometimes colloquially "z-drugs" since many of their names begin with a "z". Like benzodiazepines they include nitrogen-heterocyclic rings; typical representatives are zopiclone and zolpidem. Beside tri- and tetracyclic antidepressants (TCA), the so-called selective serotonin noradrenalin reuptake inhibitors (SSNRI) are also prescribed as antidepressants. TCA are built of similar tri- or tetracyclic structures always containing a basic nitrogen atom, while SSNRI are a heterogeneous group of active pharmaceutical ingredients. Antipsychotics are drugs used for therapy of psychosis-like delusions, hallucinations, or paranoia. These are also a rather heterogeneous group of compounds. In the context of prescription drugs, methadone is also of interest, it is dispensed under medical supervision in opioid replacement therapies. Co-consumption of sedatives and other prescription drugs is observed under replacement therapy in patients seeking to further dampen potential withdrawal symptoms and may need to be monitored to assess therapeutic success. This is one possible application of the analysis method described in this work.

Cases of driving under the influence of drugs (DUID) are another

GERSTEL AppNote 236

major area for which psychoactive compounds should be determined in blood. Driver impairment can be ascertained and causes of accidents can be elucidated. Moreover, intoxications in clinical or forensic contexts can be discovered. When these substances are used in drug-facilitated crimes and, for example, given as "knockout drops" the victims' blood must be examined. Scientific literature (1–9) reveals that benzodiazepines and other prescription drugs are normally extracted by liquid–liquid extraction (LLE) or solid-phase extraction (SPE) from serum or plasma, most often following a protein precipitation step.

Resulting extracts are routinely analyzed by LC–MS/MS or, after a derivatization step, by GC–MS(/MS). With the availability of more sensitive mass spectrometers, protein precipitation without further cleanup or enrichment has been used for LC–MS/MS determination of the aforementioned compounds.

The comprehensively automated SPE–LC–MS/MS method described in this work was developed at the Institute of Legal Medicine in Cologne, Germany, by automating a manual SPE workflow from the Institute of Legal Medicine in Münster, Germany.

Experimental

A MultiPurpose Sampler (MPS, GERSTEL) was used for automation of the sample preparation steps and injection of the final extract into the LC–MS/MS system.

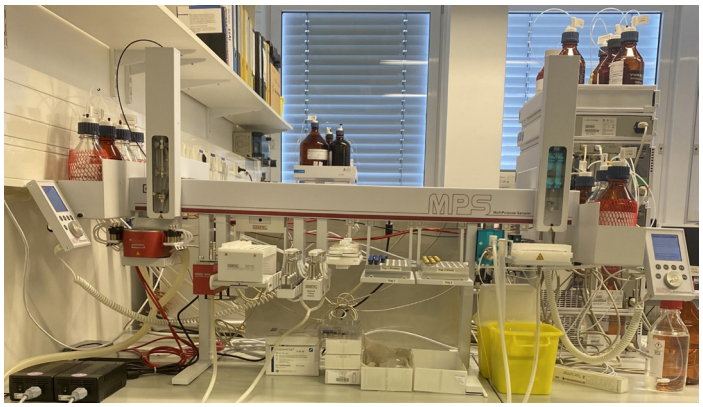

Figure 1: System for automated solid-phase extraction of psychoactive drugs in biological fluids and tissue combined with injection of the prepared extract to the LC–MS/MS system. The system is used in routine analysis at the Institute of Legal Medicine in Cologne, Germany. Modules attached to the rail from left to right: mVAP evaporation station, SPE station, SPE cartridge tray, two Solvent Filling Stations, solvent reservoir, two trays for samples and extracts, LC injection valve.

The automated system is shown in Figure 1., consisting of a Dual Head MPS (GERSTEL) with a 2.5-mL syringe on the left head for all sample preparation steps and a 100-µL syringe for injection of the prepared sample extract into the LC–MS/MS (1200 SL LC and 6460 MS, Agilent Technologies). The MPS was configured with modules for SPE and extract evaporation, as well as with solvent reservoirs. Initially a 250 µL serum or plasma sample was added to a 2 mL vial and mixed with 25 µL of an internal standard (ISTD) solution. In case of a heavily hemolytic or post-mortem specimen a manual filtration (syringe filters, 25 mm, 5 µm, Pall Acrodisc® Versapor) of diluted (pH 6 buffer) samples was performed prior to the SPE.

Sample vials were then placed in the autosampler and all further preparation steps, including injection to the analysis system, were executed automatically. Since all the included compounds can be protonated at acidic pH values, a cation exchange sorbent was chosen for analyte enrichment and cleanup (Chromabond HR-XC, 60 mg, 45 µm, 3 mL format, REF 730956P45MPS, Macherey-Nagel). The SPE automation relies on standard SPE cartridges that are cut and equipped with a transport adapter at the top and a disposable canula on the Luer port at the bottom (Figure 2).

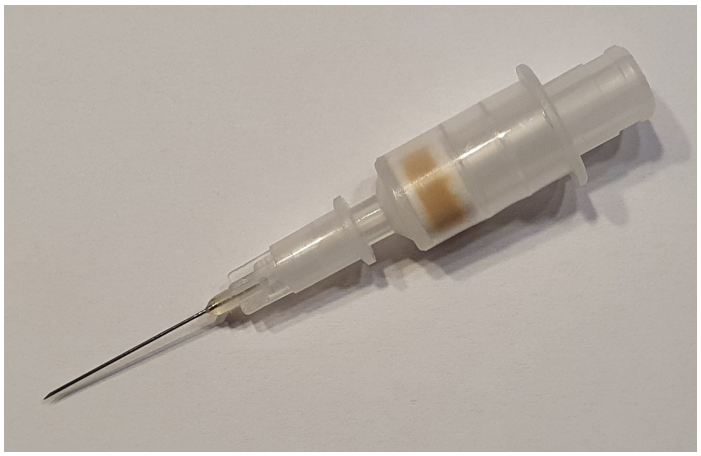

Figure 2: Standard SPE cartridge cut and equipped with a transport adapter at the top and a disposable canula on the Luer port at the bottom filled with strong cation exchange sorbent for use with the automated system in figure 1. Available as assembly: Chromabond HR-XC, 60 mg, 45 µm, 3 mL format, REF 730956P45MPS, Macherey-Nagel.

GERSTEL AppNote 236

The cartridge was conditioned by injecting 1 mL each of methanol, water, and phosphate buffer pH 6. The sample was diluted with 1 mL of phosphate buffer pH 6 (except for heavily hemolytic or post-mortem specimens, see above), loaded onto the SPE cartridge, and analytes were bound by the nonpolar backbone of the sorbent material followed by washing steps with 1 mL phosphate buffer and 70:30 (v/v) water–methanol. During these steps, medium-polar and polar interferences were washed away. In the next step the cartridge was washed with 0.1 M HCl protonating the analytes and binding them to the cation exchange groups of the sorbent. A sequence of washing steps, including methanol, followed in order to further remove interfering compounds bound to the nonpolar backbone of the sorbent while the analytes were bound to the cation exchange groups. In between these steps and directly before analyte elution, the cartridge was dried in a stream of nitrogen delivered through the 2.5-mL syringe, which was connected to a gas supply. Compounds of interest were eluted two times with 500 µL of a mixture of ethyl acetate and ammonia. The eluates were collected in vials fitted with septum caps and were subsequently evaporated to dryness in the evaporation module for 10.5 min at 50 °C under shaking and reduced pressure comparable to a rotary evaporator. The extracts were reconstituted in 100 µL of a 85:15 (v/v) water–acetonitrile mixture containing 0.1% formic acid. A 30 µL aliquot of this solution was injected into a 5 µL sample loop for transfer onto the LC column, a 150 × 2 mm, 2.7 µm Nucleoshell RP 18 (Macherey-Nagel). Analytes were separated during a 15-min gradient run with a flow of 0.3 mL/min employing water and acetonitrile, both including 0.1% formic acid, and detected in dynamic multiple reaction monitoring mode (MRM). For each target compound two MRM transitions were chosen, one quantifier and one qualifier, in addition to one MRM transition for deuterated internal standards (ISTD) used for quantification.

Results and Discussion

The analysis method was completely validated according to guidelines of the GTFCh (10, Appendix B) for 52 of the 65 target analytes (Table 1). Eight further compounds were considered as "semi-quantitative" because the complete validation data had not yet been collected. For an additional five compounds a "qualitative" detection can be performed by having established a cut-off value, which includes evaluation of extraction recovery and matrix effects. With regard to "semi-quantitative" and "qualitative" compounds, quantitation is not required on a regular basis at the laboratory in Cologne. Validation comprised establishing limits of quantification/detection (LOQ/LOD) and the linear range. Furthermore, precision (repeatability and time-different intermediate precision) and accuracy were tested at low and high concentrations inside the linear range as well as the extraction recovery, matrix effects and selectivity. Limits of quantification were between 0.76–35 ng/mL, and were below 10 ng/mL for the vast majority of compounds. The linear ranges reached upper values between 100–5000 ng/mL. For practical reasons, in many cases the lowest calibration point was chosen slightly above the LOQ to facilitate the preparation of calibration solutions (e.g. amisulprid compare Table 1). Precision data expressed as relative standard deviation were mainly below 8% and often even below 5%, proving the excellent repeatability of such an automated analysis method. Spiked blank matrix was used for calibration, blank matrix spiked with internal standard only as matrix blank sample, and external quality control samples were analyzed in every sequence. In addition, the laboratory successfully participated in "round robin" tests for the following compound classes: benzodiazepines, opioids, z-drugs, tricyclic antidepressants, and selective serotonin noradrenalin reuptake inhibitors. The developed method is included in the DIN EN ISO/IEC 17025:2018 accreditation of the forensic toxicology laboratory at the Institute of Legal Medicine in Cologne and it is routinely used for serum, plasma, whole blood, and post-mortem specimens. Over 2400 samples have been analyzed to date.

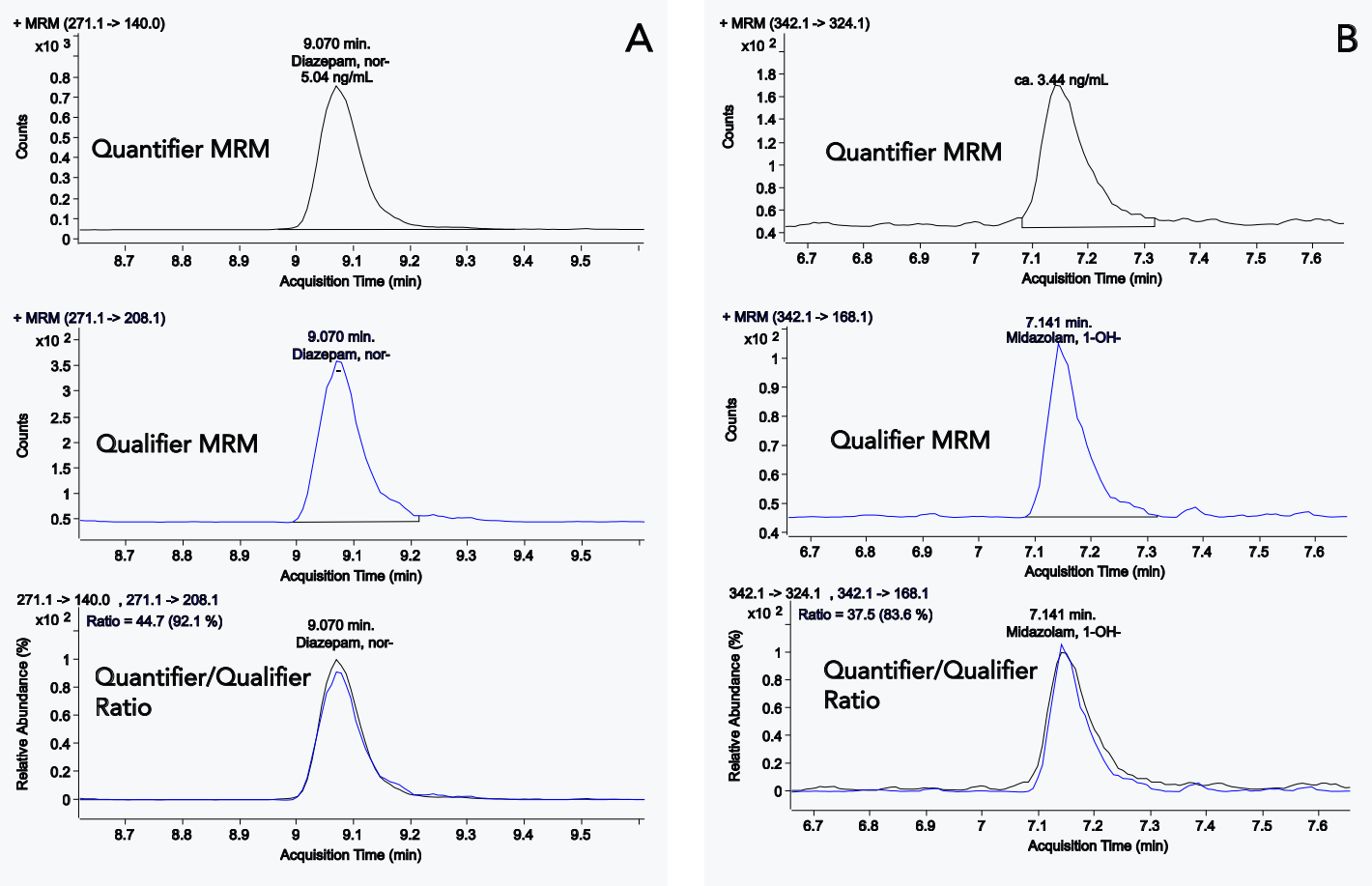

Figure 3: Example chromatograms of real samples at low analyte concentrations. Quantifier MRM traces, qualifier MRM traces and quantifier/qualifier ratio for: A Nordiazepam at 5.04 ng/mL (>LOQ) and B Midazolam, 1-hydroxy- at ca. 3.44 ng/mL (>LOD and <LOQ).

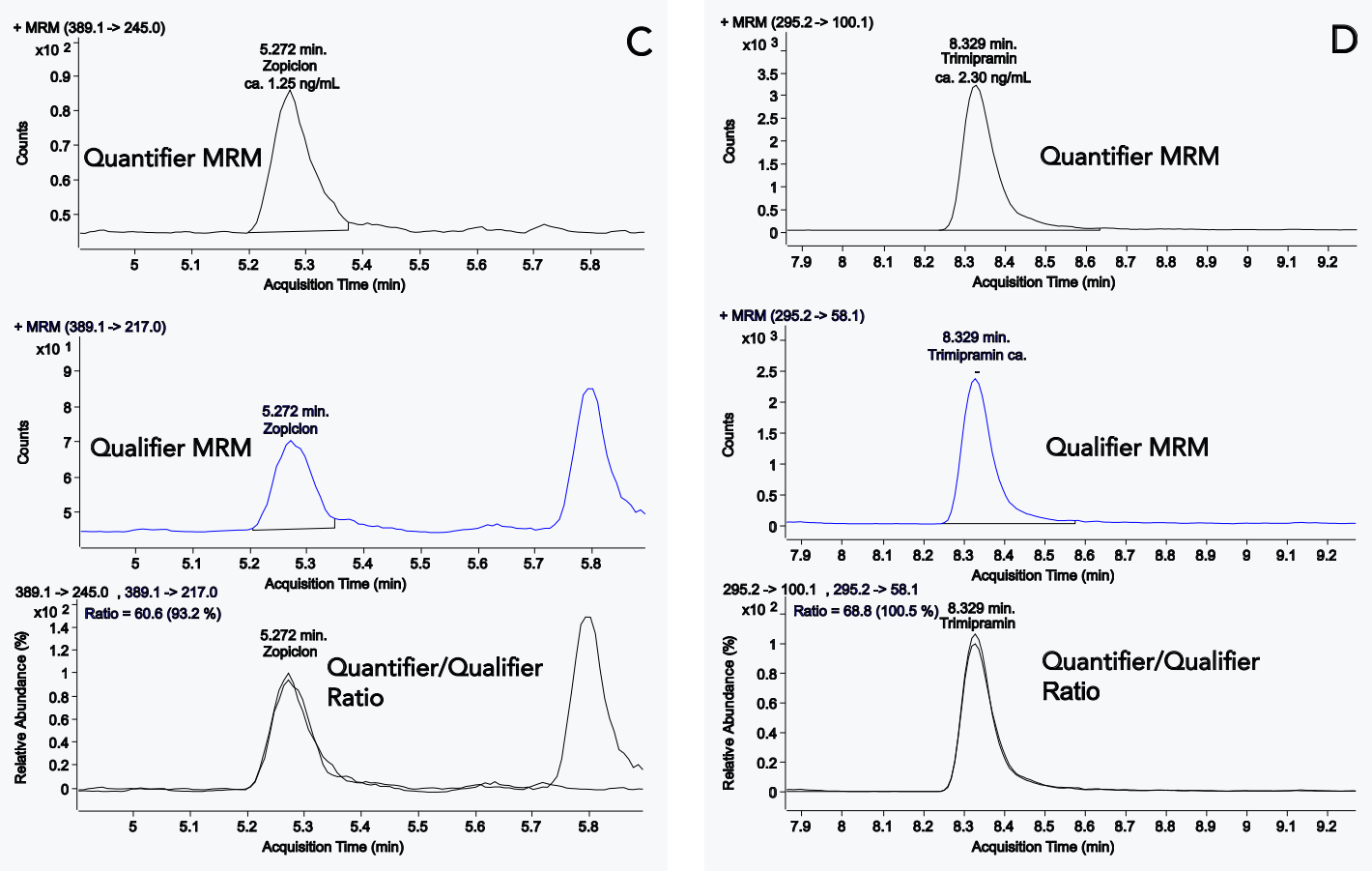

Figure 3 (cont.): Example chromatograms of real samples at low analyte concentrations. Quantifier MRM traces, qualifier MRM traces and quantifier/qualifier ratio for: C Zopiclon at ca. 1.25 ng/mL (>LOD and <LOQ) and D Trimipramin at ca. 2.30 ng/mL (>LOD and <LOQ).

Table 1: Compound list with 65 analytes, drug classes, assigned internal standards and validation data according to GTFCh guidelines [10].
RSDr: Repeatability; RSD(T): time-different intermediate precision. EDDP: 2-ethyliden-1,5-dimethyl-3,3-diphenyl-pyrrolidine; TCA: Tri- or tetracyclic antidepressant; SSNRI: Selective serotonin noradrenalin reuptake inhibitor.

Analyte	Drug Class	ISTD for quantitation	Validation	Calibration Range [ng/mL]	LOD / LOQ [ng/mL]	RSDr low / high [%] RSD(T) low / high [%] Accuracy low / high [%]	Recovery low / high [%] Matrix effect low / high [%]
Alprazolam	Benzodiazepine	Alprazolam-d5	Quantitative	2.5 – 100	1.3 / 2.5	3.5 / 3.0 3.5 / 7.4 -8.4 / -10.0	54 / 57 125 / 101
Alprazolam, 1-hydroxy-	Benzodiazepine metabolite	Alprazolam-d5	Quantitative	2.5 – 100	1.3 / 2.5	4.6 / 3.4 6.6 / 4.9 -6.1 / -9.6	46 / 39 122 / 99
Amisulprid	Antipsychotic	Nortriptylin-d3	Quantitative	5 - 500	0.87 / 2.7	12.8 / 6.7 12.8 / 10.9 -0.8 / -6.8	65 / 65 112 / 98
Amitriptylin	TCA	Amitriptylin-d3	Quantitative	5 - 500	1.2 / 2.9	9.1 / 5.2 9.6 / 8.7 -7.0 / -3.1	78 / 72 119 / 105
Aripiprazole	Antipsychotic	Aripiprazol-d8	Quantitative	5 - 500	2.3 / 4.4	3.5 / 3.0 6.4 / 3.9 9.2 / 13.7	58 / 71 108 / 85
Bromazepam	Benzodiazepine	Bromazepam-d4	Quantitative	50 – 500	11 / 29	4.5 / 6.7 / -6.0/	83 / 82 81 / 77
Bromazepam, 3-hydroxy-	Benzodiazepine metabolite	Bromazepam-d4	Semi-quantitative	10 – 100	5.0 / 10	- / - - / - - / -	72 / 68 93 / 85
Brotizolam	Benzodiazepine	Flunitrazepam-d7	Semi-quantitative	2.5 – 100	1.2 / 1.5	6.2 / 6.9 6.5 / 7.6 19.8 / 18.0	84 / 79 105 / 101
Buspiron	Anxiolytic	Zolpidem-d6	Semi-quantitative	10 – 100	5.0 / 10	- / - - / - - / -	64 / 68 88 / 94
Carbamazepine	Anticonvulsant, mood stabilizer	Carbamazepin-d10	Quantitative	50 - 5000	13 / 30	5.1 / 3.5 7.4 / 11.3 4.3 / -3.5	59 / 68 112 / 98

Table 1 (cont.): Compound list with 65 analytes, drug classes, assigned internal standards and validation data according to GTFCh guidelines [10]. RSDr: Repeatability; RSD(T): time-different intermediate precision. EDDP: 2-ethyliden-1,5-dimethyl-3,3-diphenyl-pyrrolidine; TCA: Tri- or tetracyclic antidepressant; SSNRI: Selective serotonin noradrenalin reuptake inhibitor.

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Carbamazepine epoxide	Carbamazepine metabolite	Carbamazepin-d10	Semi-quantitative	50 - 5000	9.0 / 26	6.6 / 7.4 8.0 / 11.4 -8.2 / -5.5	15 / 17 109 / 96
Citalopram	Antidepressant SSNRI	Citalopram-d6	Quantitative	5 - 500	1.0 / 2.8	7.4 / 2.4 9.0 / 9.6 2.2 / 4.1	63 / 70 118 / 97
Clobazam	Benzodiazepine	Temazepam-d5	Quantitative	25 – 500	5.8 / 18	4.7 / 8.7 5.9 / 14.2 12.0 / 11.7	78 / 79 112 / 98
Clomethiazole	Sedative, hypnotic, anticonvulsant	-	Qualitative	-	5.0 / -	- / - - / - - / -	72 / - 62 / -
Clonazepam	Benzodiazepine	Clonazepam-d4	Quantitative	2.5 – 100	1.3 / 2.5	1.4 / 5.9 3.2 / 5.9 0.1 / -1.1	92 / 80 91 / 94
Clonazepam, 7-amino-	Benzodiazepine metabolite	7-Aminoflunitrazepam-d7	Quantitative	2.5 – 100	0.44 / 1.6	4.4 / 3.5 12.1 / 10.4 -2.1 / -6.5	71 / 68 102 / 101
Clozapine	Antipsychotic	Clozapin-d4	Quantitative	5 - 500	1.1 / 3.5	7.1 / 1.5 9.1 / 6.8 1.0 / -5.0	73 / 66 123 / 110
Clozapine, nor-	Antipsychotic metabolite	Amitriptylin-d3	Quantitative	5 - 500	0.85 / 2.6	6.9 / 7.0 7.9 / 7.6 11.1 / -3.8	61 / 73 92 / 98
Diazepam	Benzodiazepine	Diazepam-d5	Quantitative	50 – 2500	11 / 35	2.4 / 3.7 3.0 / 6.2 0.8 / 2.6	85 / 83 91 / 90
Diazepam, nor-	Benzodiazepine / benzodiazepine metabolite	Diazepam-d5	Quantitative	10 – 500	2.4 / 4.5	3.6 / 4.7 6.9 / 6.8 6.7 / 2.2	88 / 83 87 / 92

Table 1 (cont.): Compound list with 65 analytes, drug classes, assigned internal standards and validation data according to GTFCh guidelines [10]. RSDr: Repeatability; RSD(T): time-different intermediate precision. EDDP: 2-ethyliden-1,5-dimethyl-3,3-diphenyl-pyrrolidine; TCA: Tri- or tetracyclic antidepressant; SSNRI: Selective serotonin noradrenalin reuptake inhibitor.

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Diphenhydramin	Antihistamine, sedative	Midazolam-d4	Semi-quantitative	10 – 100	5.0 / 10	- / - - / - - / -	66 / 68 73 / 90
Doxepin	TCA	Doxepin-d3	Quantitative	5 - 500	1.0 / 2.3	10 / 6.7 10 / 8.2 4.1 / 2.4	70 / 71 123 / 104
Doxepin, desmethyl-	TCA metabolite	Nortriptylin-d3	Quantitative	5 - 500	1.1 / 2.3	5.2 / 3.3 8.8 / 8.7 10.1 / 9.2	64 / 70 98 / 94
Flunitrazepam	Benzodiazepine	Flunitrazepam-d7	Quantitative	2.5 – 100	0.39 / 1.4	5.3 / 5.1 6.1 / 6.7 -10.5 / -11.6	90 / 82 95 / 94
Flunitrazepam, desmethyl-	Benzodiazepine	Flunitrazepam-d7	Quantitative	1 – 100	0.66 / 1.0	3.9 / 6.0 6.1 / 6.8 2.4 / -8.5	88 / 80 91 / 97
Flunitrazepam, 7-amino-	Benzodiazepine metabolite	7-Aminoflunitrazepam-d7	Quantitative	2.5 – 100	0.68 / 2.0	4.0 / 2.5 7.5 / 9.6 -0.4 / -0.6	68 / 68 100 / 105
Fluoxetin	Antidepressant SSNRI	Fluoxetin-d6	Quantitative	5 - 500	1.6 / 2.5	7.7 / 6.9 7.7 / 9.4 7.7 / 6.4	68 / 79 114 / 89
Fluoxetin, nor-	Antidepressant SSNRI metabolite	Norfluoxetin-d6	Quantitative	5-500	2.2 / 3.1	1.8 / 11.1 7.9 / 16.5 -18.5 / -14.2	69 / 67 85 / 90
Flupirtine	Analgesic	Risperidon-d4	Semi-quantitative	50 - 5000	11 / 29	- / - - / - - / -	73 / 70 122 / 104
Flurazepam	Benzodiazepine	-	Qualitative	-	5.0 / -	- / - - / - - / -	69 / 75 83 / 95

Table 1 (cont.): Compound list with 65 analytes, drug classes, assigned internal standards and validation data according to GTFCh guidelines [10]. RSDr: Repeatability; RSD(T): time-different intermediate precision. EDDP: 2-ethyliden-1,5-dimethyl-3,3-diphenyl-pyrrolidine; TCA: Tri- or tetracyclic antidepressant; SSNRI: Selective serotonin noradrenalin reuptake inhibitor.

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Flurazepam, desalkyl-	Benzodiazepine metabolite	Temazepam-d5	Quantitative	10 – 500	1.8 / 9.6	5.6 / 8.1 5.9 / 8.6 5.4 / 5.7	84 / 82 90 / 92
Fluvoxamin	Antidepressant SSNRI	Fluoxetin-d6	Quantitative	5 - 500	0.95 / 3.9	4.8 / 1.9 7.2 / 4.9 9.0 / 12.2	78 / 71 122 / 117
Lorazepam	Benzodiazepine	Oxazepam-d5	Quantitative	10 – 500	2.4 / 5.9	4.5 / 3.8 4.5 / 7.5 -10.6 / -6.6	90 / 83 92 / 97
Lormetazepam	Benzodiazepine	Temazepam-d5	Quantitative	2.5 – 100	0.96 / 1.6	3.5 / 4.3 3.9 / 5.8 8.7 / 14.0	91 / 81 115 / 107
Medazepam	Benzodiazepine	Midazolam-d4	Quantitative	25 – 500	5.1 / 23	5.1 / 4.2 6.0 / 7.2 4.9 / 6.5	80 / 82 88 / 85
Methadone	Opioid	Methadone-d9	Quantitative	25 – 500	6.8 / 21	4.7 / 5.2 12.9 / 7.9 -6.8 / -4.2	69 / 76 88 / 89
EDDP	Methadone metabolite	EDDP-d3	Quantitative	25 – 500	6.3 / 18	4.6 / 4.2 10.7 / 8.6 3.9 / 2.1	63 / 69 106 / 97
Midazolam	Benzodiazepine	Midazolam-d4	Quantitative	10 – 500	2.9 / 8.6	2.7 / 4.6 2.8 / 4.6 -3.8 / -1.1	69 / 72 84 / 94
Midazolam, 1-hydroxy-	Benzodiazepine metabolite	Midazolam-d4	Quantitative	20 – 200	2.1 / 7.4	4.4 / 8.4 4.4 / 8.4 3.6 / 10.4	69 / 72 96 / 92
Mirtazapin	TCA	Clozapin-d4	Quantitative	5 - 500	1.3 / 3.5	10.6 / 5.1 12.8 / 8.8 -5.8 / -9.0	88 / 70 123 / 117

Table 1 (cont.): Compound list with 65 analytes, drug classes, assigned internal standards and validation data according to GTFCh guidelines [10]. RSDr: Repeatability; RSD(T): time-different intermediate precision. EDDP: 2-ethyliden-1,5-dimethyl-3,3-diphenyl-pyrrolidine; TCA: Tri- or tetracyclic antidepressant; SSNRI: Selective serotonin noradrenalin reuptake inhibitor.

Analyte	Drug Class	ISTD for quantitation	Validation	Calibration Range [ng/mL]	LOD / LOQ [ng/mL]	RSDr low / high [%] RSD(T) low / high [%] Accuracy low / high [%]	Recovery low / high [%] Matrix effect low / high [%]
Nitrazepam	Benzodiazepine	Flunitrazepam-d7	Quantitative	10 – 500	2.1 / 2.7	3.6 / 5.3 5.8 / 5.3 -6.5 / -6.4	86 / 77 92 / 96
Nitrazepam, 7-amino-	Benzodiazepine metabolite	-	Qualitative	-	5.0 / -	- / - - / - - / -	- / 27 - / 93
Nortriptylin	TCA, metabolite amitriptylin	Nortriptylin-d3	Quantitative	5 - 500	0.77 / 2.3	5.9 / 2.2 5.9 / 5.4 10.7 / 11.3	64 / 71 117 / 94
Olanzapine	Antipsychotic	Olanzapin-d8	Quantitative	5 - 500	1.2 / 3.7	7.6 / 2.6 7.6 / 7.9 9.4 / 7.0	58 / 71 112 / 112
Opipramol	TCA	Clozapin-d4	Quantitative	5 - 500	2.5 / 5.0	6.9 / 2.6 9.5 / 7.5 2.7 / -11.0	60 / 79 103 / 97
Oxazepam	Benzodiazepine / benzodiazepine metabolite	Oxazepam-d5	Quantitative	50 – 2500	8.3 / 25	3.9 / 3.5 7.2 / 7.4 -2.0 / -4.4	85 / 81 93 / 96
Paroxetin	Antidepressant SSNRI	Nortriptylin-d3	Quantitative	5 - 500	1.0 / 3.0	4.6 / 5.1 4.6 / 5.1 12.8 / 14.0	60 / 71 124 / 100
Perphenazin	Antipsychotic	-	Qualitative	-	2.0 / -	- / - - / - - / -	- / 64 - / 42
Promethazine	Antihistamine, sedative	Nortriptylin-d3	Semi-quantitative	5 - 500	2.5 / 5.0	4.7 / 2.7 4.7 / 8.5 4.5 / 2.0	- / - - / -
Quetiapin	Antipsychotic	Doxepin-d3	Quantitative	5 - 500	1.1 / 4.0	8.5 / 4.7 8.5 / 8.9 4.9 / 4.2	73 / 67 117 / 107

Table 1 (cont.): Compound list with 65 analytes, drug classes, assigned internal standards and validation data according to GTFCh guidelines [10]. RSDr: Repeatability; RSD(T): time-different intermediate precision. EDDP: 2-ethyliden-1,5-dimethyl-3,3-diphenyl-pyrrolidine; TCA: Tri- or tetracyclic antidepressant; SSNRI: Selective serotonin noradrenalin reuptake inhibitor.

Analyte	Drug Class	ISTD for quantitation	Validation	Calibration Range [ng/mL]	LOD / LOQ [ng/mL]	RSDr low / high [%] RSD(T) low / high [%] Accuracy low / high [%]	Recovery low / high [%] Matrix effect low / high [%]
Risperidone	Antipsychotic	Risperidon-d4	Quantitative	5 - 500	1.6 / 3.8	7.9 / 4.0 7.9 / 9.3 4.4 / 4.2	83 / 75 116 / 97
Paliperidone (Risperidone, 9-hydroxy-)	Antipsychotic / anti-psychotic metabolite	Doxepin-d3	Quantitative	5 - 500	1.1 / 3.9	3.5 / 3.2 5.7 / 13.3 12.3 / -0.7	64 / 62 127 / 114
Sertralin	Antidepressant SSNRI	Sertralin-d3	Quantitative	10 - 500	1.9 / 6.2	10.6 / 4.6 10.6 / 8.0 1.7 / 0.5	63 / 76 101 / 78
Temazepam	Benzodiazepine / benzodiazepine metabolite	Temazepam-d5	Quantitative	10 – 500	4.3 / 6.5	3.0 / 4.0 4.7 / 7.1 3.6 / 0.2	88 / 78 97 / 99
Tetrazepam	Benzodiazepine	Diazepam-d5	Quantitative	10 – 500	4.0 / 6.2	2.0 / 3.3 2.0 / 3.3 13.4 / 7.6	79 / 71 80 / 85
Tetrazepam, nor-	Benzodiazepine metabolite	Diazepam-d5	Semi-quantitative	10 – 100	5.0 / 10	- / - - / - - / -	66 / 70 68 / 94
Triazolam	Benzodiazepine	Flunitrazepam-d7	Quantitative	1 – 100	0.58 / 0.76	3.8 / 8.0 6.4 / 8.0 -3.8 / -1.9	76 / 71 103 / 103
Triazolam, 1-hydroxy-	Benzodiazepine metabolite	Midazolam-d4	Quantitative	2.5 – 100	1.3 / 2.5	3.1 / 7.2 4.9 / 9.8 -15.5 / -10.8	73 / 69 127 / 102
Trimipramin	TCA	Trimipramin-d3	Quantitative	5 - 500	1.2 / 4.0	7.0 / 2.5 9.9 / 6.6 -0.7 / 0.8	76 / 79 120 / 98
Venlafaxin	Antidepressant SSNRI	Venlafaxin-d6	Quantitative	5 - 500	0.93 / 2.3	7.4 / 2.3 7.5 / 8.5 4.7 / 4.5	72 / 73 122 / 100

Table 1 (cont.): Compound list with 65 analytes, drug classes, assigned internal standards and validation data according to GTFCh guidelines [10]. RSDr: Repeatability; RSD(T): time-different intermediate precision. EDDP: 2-ethyliden-1,5-dimethyl-3,3-diphenyl-pyrrolidine; TCA: Tri- or tetracyclic antidepressant; SSNRI: Selective serotonin noradrenalin reuptake inhibitor.

Analyte	Drug Class	ISTD for quantitation	Validation	Calibration Range [ng/mL]	LOD / LOQ [ng/mL]	RSDr low / high [%] RSD(T) low / high [%] Accuracy low / high [%]	Recovery low / high [%] Matrix effect low / high [%]
Venlafaxin, O-desmethyl-	SSNRI metabolite	Doxepin-d3	Quantitative	5 - 500	1.2 / 1.9	6.5 / 8.3 9.8 / 11.0 5.7 / -4.9	67 / 71 112 / 95
Zaleplon	Sedative, hypnotic (Z-drug)	Clonazepam-d4	Quantitative	2.5 – 100	0.60 / 1.9	3.0 / 7.5 3.4 / 7.8 9.6 / 4.0	68 / 86 105 / 101
Zolpidem	Sedative, hypnotic (Z-drug)	Zolpidem-d6	Quantitative	10 – 500	2.1 / 7.9	2.9 / 3.6 4.5 / 3.6 0.6 / -0.1	70 / 85 91 / 96
Zopiclone	Sedative, hypnotic (Z-drug)	Zolpidem-d6	Quantitative	2.5 – 100	0.62 / 1.7	4.0 / 3.7 13.5 / 13.8 6.6 / -2.6	66 / 70 115 / 116
2-Amino-5-chloro pyridine (ACP)	Decomposition product of zopiclone	-	Qualitative	-	5.0 / -	- / - - / - - / -	57 / - - / -

GERSTEL AppNote 236

A few compounds of interest cannot be detected by immunochemical pre-testing, and therefore the described SPE–LC–MS/MS method is used as a multi-targeted screening that includes confirmation analysis and quantitation, as needed. Before implementation of this method the parameters were contained in two separate workflows running on a standalone SPE extractor. Each of them needed 0.5 mL of sample resulting in a total required volume of 1 mL, while today only 0.25 mL is needed for the complete set of analytes. The former methods demanded supervision and several manual interventions, such as evaporation of the extracts and reconstitution, requiring undivided attention of laboratory personnel, making it difficult to perform other tasks. Comprehensive automation of the workflow, including LC injection, reduces the workload and gives lab personnel time for other important tasks, such as data review or developing new analysis methods. Due to the extensive cleanup and enrichment procedure, good quality chromatograms with very low levels of interfering compounds are achieved (Figure 3) and the maintenance effort for the LC–MS/MS instrument is reduced. Analyte peaks can be identified unambiguously and automated integration results in accurate quantification without the need for reintegration for the vast majority of peaks.

Conclusions

The presented comprehensive automation of complex sample preparation workflows, including the injection into an analysis system, greatly reduces manual laboratory workload. Data quality is high without depending on manual intervention and reintegration. In addition to the application described in this work, the forensic toxicology laboratory in Cologne also employs a similar setup coupled to a GC–MS/MS system for the automated determination of cannabinoids and metabolites in plasma/serum, urine and hair. These examples illustrate the benefits of implementing automated sample preparation methods into laboratory workflows.

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