

Detection and identification of 700 drugs by multi-target screening with a 3200 Q TRAP[®] LC-MS/MS system and library searching

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Abstract The multi-target screening method described in this work allows the simultaneous detection and identification of 700 drugs and metabolites in biological fluids using a hybrid triple-quadrupole linear ion trap mass spectrometer in a single analytical run. After standardization of the method, the retention times of 700 compounds were determined and transitions for each compound were selected by a “scheduled” survey MRM scan, followed by an information-dependent acquisition using the sensitive enhanced product ion scan of a Q TRAP[®] hybrid instrument. The identification of the compounds in the samples analyzed was accomplished by searching the tandem mass spectrometry (MS/MS) spectra against the library we developed, which contains electrospray ionization–MS/MS spectra of over 1,250 compounds. The multi-target screening method together with the library was included in a software program for routine screening and quantitation to

achieve automated acquisition and library searching. With the help of this software application, the time for evaluation and interpretation of the results could be drastically reduced. This new multi-target screening method has been successfully applied for the analysis of postmortem and traffic offense samples as well as proficiency testing, and complements screening with immunoassays, gas chromatography–mass spectrometry, and liquid chromatography–diode-array detection. Other possible applications are analysis in clinical toxicology (for intoxication cases), in psychiatry (antidepressants and other psychoactive drugs), and in forensic toxicology (drugs and driving, workplace drug testing, oral fluid analysis, drug-facilitated sexual assault).

Keywords Drug monitoring · Drug screening · Forensics · Toxicology · Mass spectrometry · Liquid chromatography–tandem mass spectrometry

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Introduction

Identification of drugs in biological fluids is currently performed with a variety of analytical techniques, including immunoassays, available only for a small number of substance classes, and chromatographic techniques such as gas chromatography (GC) coupled with mass spectrometry (MS) [1] and liquid chromatography (LC) with ultraviolet (UV) detection [2]. Although these techniques are well established, widely used, and offer comprehensive spectral libraries, they suffer from some limitations, including laborious and time-consuming derivatization steps for the analysis of nonvolatile and polar drugs by GC-MS. LC is ideally suited for polar compounds, but UV detection does not allow the evaluation of overlapping peaks of coeluted compounds.

In the last few years, LC with MS detection has been increasingly used for toxicological screening, and different types of instruments and strategies have been applied. High mass resolution mass spectrometers such as time-of-flight (TOF) instruments have been used to identify analytes by accurate mass detection and the assignment to a molecular formula [3] and by a further enhancement of identification by including the isotopic pattern of the compounds [4].

Other strategies require the generation of mass-spectral libraries to identify compounds in biological samples which were created with single-stage mass spectrometers by “in source” collision-induced dissociation with an inert collision gas [5, 6] or by collision-induced dissociation in a collision cell after isolating the precursor ions to create background-free MS/MS spectra, which were recorded using quadrupoles [7], linear ion traps [8–11], or TOF mass analyzers [12, 13].

Typically, LC screening methods with MS/MS identification use a survey scan to detect analytes and a dependent scan providing the MS/MS spectra that undergo a library search for identification. The dependent scan can be accomplished in a second analytical run [14] or a data-dependent acquisition [15] or an information-dependent acquisition (IDA) [9, 10, 12, 16–18] is used to automatically select precursor ions as soon they have been detected with the survey scan within the same analytical run. While general unknown screening procedures can be performed with full-scan techniques such as a Q1 scan, an enhanced MS scan with a linear ion trap [10, 16, 17] or TOF-MS [19, 20] as the survey scan, our approach is a multi-target screening (MTS) with a sensitive and selective multiple reaction monitoring (MRM) for detection. For identification, the MS/MS spectra obtained by the IDA controlled enhanced product ion (EPI) scans are compared with the spectra of our electrospray ionization–MS/MS library which was previously published [8].

Compared with our former MTS [9], the method presented here covers more target analytes, which could be achieved with scheduled MRM (sMRM) transitions without increasing the MRM cycle time. sMRM is used to analyze MRM transitions only at the retention times of the corresponding analytes within a defined time window. In this way, fewer MRM transitions have to be monitored in one cycle.

Furthermore, the IDA parameters were optimized to enhance the identification of coeluted compounds: the second most intense MRM signal of a cycle is also considered for the dependent scan and as soon as one MRM transition triggers a dependent scan twice in two consecutive cycles, the corresponding precursor ion is excluded from the dependent scan for 15 s. Although more drugs can be detected, the total cycle time of the previous method could be maintained by recording only one EPI

scan with a collision energy spread (CES) of 35 ± 15 eV instead of three spectra with collision energies of 20, 35, and 50 eV. The acquisition of CES spectra for which the linear ion trap is filled with product ions generated by collision energies of 20, 35 and 50 eV prior to detection can drastically reduce the duty cycle of an IDA screening method, since the acquisition time for a CES spectrum is as long as the time for one spectrum with a single collision energy. However, the assignment of each fragmentation pattern to a single energy level is lost.

Another analytical column and an optimized gradient shortens the run time of the method and also its integration together with the MS/MS library into the Cliquid® 2.0 software program, which works in conjunction with Analyst® (both Applied Biosystems/MDS Sciex) and enables automated acquisition, evaluation of the data obtained, and report generation. The time reduction achieved is over 20 min per sample.

Experimental

Reagents, standards, and samples

The internal standards (doxepin- d_3 and diazepam- d_5) were purchased from Promochem/Radian (Wesel, Germany). The system suitability test mixture containing amiodarone, amphetamine, caffeine, codeine, diazepam, doxepin, haloperidol, and morphine was provided by Restek (Bad Homburg, Germany). All solvents were of analytical grade. High-performance LC (HPLC)-grade methanol, acetonitrile, and formic acid (analytical grade) were obtained from Merck (Darmstadt, Germany). Ammonium formate (analytical grade) was from Sigma (Deisenhofen, Germany). Deionized water was prepared with a cartridge deionizer from Memtech (Moorenweis, Germany). Blank serum and urine samples were obtained from the University Hospital of Freiburg (Freiburg, Germany).

Sample preparation

For a fast and nonselective sample preparation, 100 μ L of urine was mixed with 100 μ L of acetonitrile containing the internal standards doxepin- d_3 and diazepam- d_5 at a concentration of 10 μ g/mL each. After vortexing and centrifugation at $16,100 \times g$, the supernatant was diluted with 800 μ L of water (equal to a dilution factor of 10). The same procedure was carried out with a dilution of the supernatant with 100 μ L of water (equal to a dilution factor of 3).

For the analysis of samples containing low concentrations of analytes, an extraction procedure was carried out before analysis: 1 mL of urine or serum sample was

extracted by alkaline liquid–liquid extraction using 0.5 mL of borate buffer (pH 9) followed by 1.5 mL of 1-chlorobutane. After 4 min of mixing and 5 min of centrifugation at $2,860\times g$, the organic supernatant was transferred to an HPLC vial and evaporated to dryness under a nitrogen stream at 40 °C. The residue was dissolved in 100 μL of mobile phase (90:10 v/v solvent A to solvent B). Solvent A was water with 0.2% formic acid and 2 mM ammonium formate and solvent B was acetonitrile with 0.2% formic acid and 2 mM ammonium formate. For cases when only a small amount of sample was available, 0.5 mL of serum was mixed with 1 mL of borate buffer (pH 9) prior to extraction.

Instrumentation and method

The LC-MS/MS system consisted of a 3200 Q TRAP[®] triple-quadrupole linear ion trap mass spectrometer fitted with a TurboIonSpray interface (Applied Biosystems/MDS Sciex, Darmstadt, Germany) and a Shimadzu Prominence HPLC system (two LC-20ADsp isocratic pumps, a CTO-20AC column oven, an SIL-20AC autosampler, a DGU-20A3 degasser, and a CBM-20A controller).

Separation was performed with a pentafluorophenyl reversed-phase column (Restek Allure PFP Propyl 50 mm \times 2.1 mm inner diameter, 5 μm particle size) with an equivalent guard column (10 mm \times 2.1 mm) (Restek, Bad Homburg, Germany) and gradient elution using solvents A and B. The gradient started with 10% of solvent B at a flow rate of 0.5 mL/min and increased within 10 min linearly to 90% at a flow rate of 1 mL/min, which was kept for 5 min. The starting conditions were restored within 0.5 min and kept for 2 min, allowing the system to reequilibrate. Thirty microliters of prepared sample was injected.

To check the LC-MS/MS system in terms of retention times and mass-spectral performance, a system suitability test mixture containing eight compounds (amiodarone, amphetamine, caffeine, codeine, diazepam, doxepin, haloperidol, and morphine) covering the chromatographic run time was used each day before starting the analysis of a batch of samples.

The method consists of a survey scan and an IDA-triggered dependent scan. As a survey scan an MRM method with 700 transitions in positive ionization mode for 700 analytes was established by using our MS/MS library and the MRM catalogue of Cliquant[®] 2.0 to automatically select the precursor mass, the most intensive product ion, and its corresponding collision energy. The MRM transitions were only analyzed at a time window of ± 60 s and the total cycle time of the MRM mode was 2.1 s including the pause time between the MRM transitions of 2 ms. The compounds with corresponding MRM transitions and retention times are shown in Table S1.

The declustering potential was 40 V, the entrance potential was 10 V, the cell entrance potential was mass-dependent, the cell exit potential was 4 V, and an ion spray voltage of 4,000 V was applied. Q1 and Q3 were used at unit resolution (0.6–0.8 amu at half height). The gas settings were as follows: curtain gas 20 psi; collision gas 6 psi; ion source gas 1, 40 psi; and ion source gas 2, 70 psi. The source temperature was set to 500 °C.

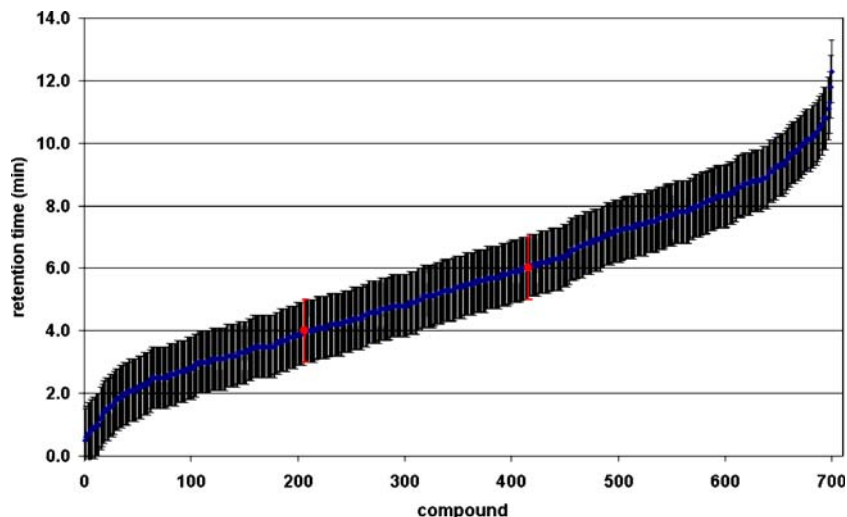
The IDA intensity threshold was set to 1,000 counts per second. The two most intense MRM transitions per cycle exceeding this threshold were considered for the dependent EPI scan. For further improvement of the identification of coeluted compounds, the MRM transitions which triggered the dependent scan twice consecutively were excluded for EPI scans for 15 s.

The EPI scans were performed at a scan range of 50 to 700 amu after a fixed fill time of 50 ms with a scan rate of 4,000 amu/s applying a CES of 35 ± 15 eV. Q0 trapping was activated to accumulate ions in Q0 while concurrently scanning ions from the linear ion trap and the pause time after the EPI scans was set to 5 ms. The declustering potential, entrance potential, gas values, and source temperature were the same as used for the MRM mode. Analyst[®] version 1.5 and Cliquant[®] 2.0 (both Applied Biosystems/MDS Sciex) were used to operate the LC-MS/MS system.

Results

The introduction of the scheduled MRM approach allowed the inclusion of more target analytes in the screening method. In sMRM mode the time the MRM transitions are monitored in one cycle (dwell time) cannot be chosen by the user. The total cycle time of the MRM experiment can be defined, whereas the dwell time is calculated by the system and depends on the number of MRM transitions that are monitored within one cycle. To ensure that the dwell times do not drop below 5 ms, which is supposed to be the minimum dwell time for an MRM transition as applied for every transition in our previous method, a closer examination of the distribution of the retention times and the applied tolerance window is essential. For this method the maximum overlapping retention time windows are 211 and lie between 4 and 6 min (see Fig. 1), which is less than the 298 MRM transitions that were monitored during the complete analytical run of our previous no scheduled MTS. Besides the advantage of more target analytes, sMRM also increases the sensitivity of the MRM mode, since dwell time of more than 5 ms is available for each transition. Furthermore, sMRM reduces the acquisition of signals caused by matrix components which can inhibit the acquisition of EPI spectra of coeluted analytes despite

Fig. 1 Distribution of the retention times of the 700 analytes covered by the multi-target screening. The retention time window for every multiple reaction monitoring (MRM) transition is indicated by the *black bars*. The maximum overlapping retention time windows occur between 4 and 6 min



exclusion parameters in the IDA settings. If the matrix component is not eluted within the retention time window of the analyte with which it is interfering, it will not be recorded.

A mixture of eight compounds was established to be used as a system suitability test. The retention times of the compounds as well as the mass spectra quality of caffeine and diazepam were verified by Cliquid® 2.0. A chromatogram of the system suitability test is shown in Fig. 2.

The sensitivity of the method was tested by spiking a serum sample with a mixture of benzodiazepines and zolpidem (mixture 1) and a urine sample with a selection of drugs of abuse and others (mixture 2). A liquid–liquid

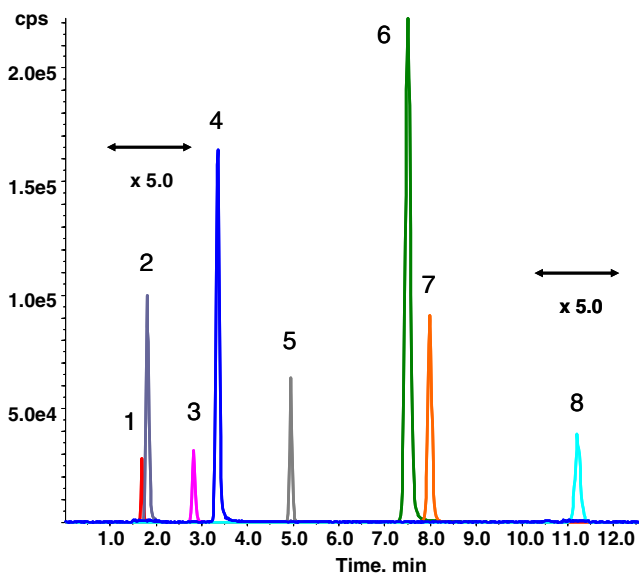


Fig. 2 MRM signals of the system suitability test. The signals for caffeine, morphine, and amiodarone have been zoomed by a factor of 5. 1 caffeine, 2 morphine, 3 codeine, 4 amphetamine, 5 diazepam, 6 doxepin, 7 haloperidol, 8 amiodarone

Table 1 Results of the analysis of a blank serum spiked with a mixture of benzodiazepines after liquid–liquid extraction with chlorobutane at pH 9. For flunitrazepam and 7-aminoflunitrazepam, lower concentrations were analyzed. The *numbers* are the purity values obtained after a library search of the enhanced product ion (EPI) spectra

Compound	Concentration (ng/mL)			
	0	10	50	100
Bromazepam	***	***	77	82
Chlordiazepoxide	***	**	86	85
Clonazepam	***	94	*** ^a	88
Diazepam	***	77	69	65
Flurazepam	***	85	94	91
Lorazepam	***	63	86	82
Midazolam	***	89	87	87
Nitrazepam	***	*	84	89
Nordiazepam	***	64	81	77
Oxazepam	***	*	71	60
Temazepam	***	82	88	76
Zolpidem	***	97	96	89
	0	1	5	10
7-Aminoflunitrazepam	***	66	82	76
Flunitrazepam	***	**	86	91

*EPI quality not sufficient for identification, ** multiple reaction monitoring (MRM) signal is positive but no acquisition of an EPI spectrum was triggered, *** no MRM signal was detected

^a The MRM signal of clonazepam was completely overlaid with the signals of nordiazepam and temazepam and no acquisition of an EPI spectrum was triggered. At 10 ng/mL, clonazepam is only partly overlaid by nordiazepam. Although both are completely covered by temazepam, the information-dependent acquisition settings allow the identification of the three compounds, since all trigger the acquisition of an EPI spectrum.

Table 2 Results of the analysis of a blank urine spiked with a mixture of drugs of abuse and others after liquid–liquid extraction with chlorobutane at pH 9. The *numbers* are the purity values obtained after a library search of the EPI spectra. Each mixture (10 and 100 ng/mL) was extracted twice

Compound	Concentration (ng/mL)			
	10 Extraction 1	10 Extraction 2	100 Extraction 1	100 Extraction 2
Carbamazepine	87	86	81	70
Diphenhydramine	**	**	90	88
Phencyclidine	98	91	89	86
Norfentanyl	*	74	89	94
Venlafaxine	61	87	80	84
Tilidine	75	78	70	65
Acetylmorphine	83	76	78	82
MDA	92	69	95	94
MDMA	90	88	93	91
Methamphetamine	*	80	80	80
Methadone	93	81	80	81
Cocaine	97	91	84	87
Codeine	73	73	70	64
Doxepin	78	89	92	94
Fentanyl	100	100	93	96
Imipramine	89	93	91	89

*EPI quality not sufficient for identification, **MRM signal is positive but no acquisition of an EPI spectrum was triggered

extraction with chlorobutane at pH 9 was performed. According to the “Workgroup Extraction” of the GTFCh (Society of Toxicological and Forensic Chemistry, Germany) [21], the extraction yields of the spiked compounds are 60% for methamphetamine, 70% for MDA and MDMA, and above 80% for the other compounds (the extraction yields of the metabolites 7-aminoflunitrazepam, acetylmorphine, and norfentanyl are not included).

The samples were analyzed using Cliquid® 2.0. Its automated data processing compared the acquired EPI

spectra with our implemented library and generated a report indicating the compounds that could be identified and the purity values after library search. In addition to the system suitability test prior to sample acquisition, the use of two internal deuterated standards allowed the control of the

Table 3 Results of the analysis of a blank urine spiked with a mixture of benzodiazepines after dilution. The *numbers* are the purity values obtained after a library search of the EPI spectra

Compound	Concentration (ng/mL)		
	1,000	100	100 ^a
Bromazepam	68	**	79
Chlordiazepoxide	78	**	*
Clonazepam	77	*	82
Diazepam	73	**	73
Flunitrazepam	88	*	71
Flurazepam	91	*	79
Lorazepam	*	**	**
Midazolam	82	65	70
Nordiazepam	77	*	*
Oxazepam	65	*	*
Temazepam	85	*	84

*EPI quality not sufficient for identification, ** MRM signal is positive but no acquisition of an EPI spectrum was triggered

^a The urine sample was only diluted 1:3 (v/v) with internal standard solution in acetonitrile and water, in contrast to the other samples, which were diluted 1:10.

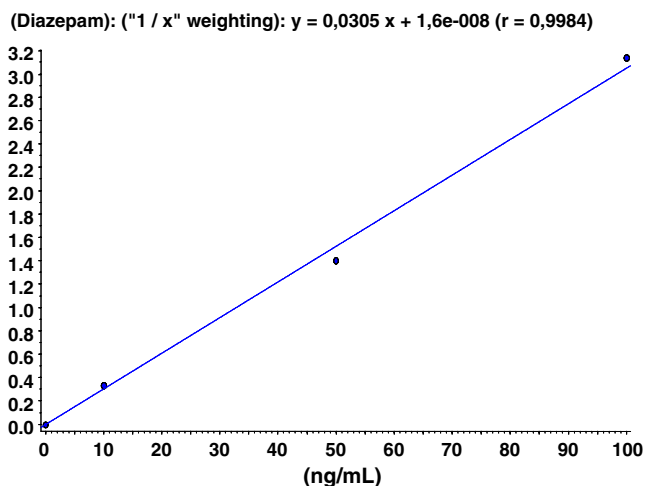


Fig. 3 Calibration curve of diazepam in serum after liquid–liquid extraction with chlorobutane at pH 9. Diazepam-*d*₅ was used as the internal standard

Table 4 Results of the analysis of a blank urine spiked with a mixture of drugs of abuse and others after dilution. The *numbers* are the purity values obtained after a library search of the EPI spectra

Compound	Concentration (ng/mL)		
	1,000	100	100 ^a
Acetylmorphine	82	**	77
Benzoylcegonine	84	87	86
Carbamazepine	92	*	*
Cocaine	95	91	94
Codeine	80	**	*
Diphenhydramine	**	**	**
Doxepin	83	*	74
Fentanyl	98	94	95
Imipramine	93	65	83
MDA	96	*	*
MDMA	84	67	73
Methadone	93	74	79
Methamphetamine	80	80	*
Norfentanyl	96	*	*
Phencyclidine	97	93	100
Tilidine	74	75	75
Venlafaxine	87	*	64

*EPI quality not sufficient for identification, ** MRM signal is positive but no acquisition of an EPI spectrum was triggered

^aThe urine sample was only diluted 1:3 (v/v) with internal standard solution in acetonitrile and water, in contrast to the other samples, which were diluted 1:10.

system performance. For quality control of the library search, the spectral purity had to exceed 70% for diazepam-*d*₅ and doxepin-*d*₃. If this requirement was not fulfilled mass spectrometer maintenance had to be performed, such as mass calibration or source cleaning.

Purity values are expressed as a percentage and were used to characterize the spectral similarity. While the fit value gives information about the similarity of the signals in the library spectrum with those in the unknown spectrum, the reverse fit value reflects the similarity of the signals in an unknown spectrum with those of a library spectrum. The purity is a combination of both values displaying the measure of the unmatched peaks between the unknown and the reference spectra. The results are shown in Table 1 for mixture 1 and in Table 2 for mixture 2.

The areas of the MRM signals of the benzodiazepines were used to establish calibration curves with diazepam-*d*₅ as an internal standard to investigate the potential of the method for semiquantitative analysis. Only for diazepam good linearity was achieved (Fig. 3), indicating that deuterated analogues have to be used for quantitative purpose. This compensates signal enhancement or suppres-

Table 5 Results of the consecutive analysis (*n*=5) of a blank urine sample spiked with a mixture of compounds after a 1:10 dilution with 100 ng/mL (*top*) and 1 µg/mL (*bottom*) of each compound

Compound	Minimum purity (%)	Maximum purity (%)	Mean purity (%)	Standard deviation (%)
Benzoylcegonine	83	90	86.4	3.05
MDMA	77	88	82.8	4.32
Diazepam	60	83	71.4	9.76
Tilidine	64	80	75.0	6.75
Midazolam	71	89	84.0	7.42
Fentanyl	95	99	97.0	1.58
Phencyclidine	84	100	91.1	5.76
Methadone	54	78	67.6	9.74
<hr/>				
Benzoylcegonine	79	85	81.8	2.68
MDMA	82	86	84.2	1.48
Diazepam	70	77	74.2	3.03
Tilidine	73	93	82.4	7.80
Midazolam	82	93	87.4	4.83
Fentanyl	90	96	94.6	2.61
Phencyclidine	95	100	97.4	2.41
Methadone	80	88	85.4	3.13

sion by matrix components or coeluted analytes, to which electrospray is susceptible [22].

Another procedure for sample preparation is dilution of urine. It delivers fastest results without loss of compounds due to more or less selective extraction procedures, which are necessary for low concentrations of analytes but are time-consuming. A urine sample was spiked with a mixture of the benzodiazepines with 1 µg/mL and 100 ng/mL of each compound. For the lower concentration, the urine was not only diluted 1:10 (v/v) with internal standard solution in methanol but also 1:3 (v/v), which could increase the number of detected and identified compounds. The results are summarized in Table 3.

The same procedure was performed with a mixture of drugs of abuse and other drugs (see Table 4).

The reproducibility of the method in terms of purity values was determined by analyzing a urine sample spiked with a mixture of eight compounds with 1 µg/mL and 100 ng/mL of each compound. Therefore, the urine was diluted 1:10 (v/v) and each sample was injected five times consecutively. The minimum purities, maximum purities, mean purities, and standard deviations are shown in Table 5.

As expected, the sample extractions allowed the detection of lower concentrations owing to a preconcentration of the analytes. However, it has to be taken into consideration that no extraction method is capable of extracting all analytes of the target screening.

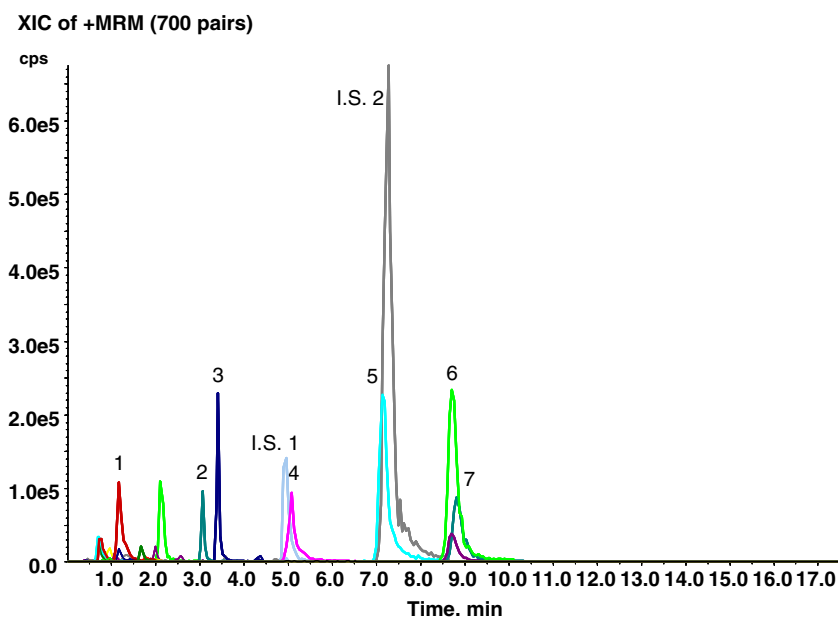


Fig. 4 Extracted MRM chromatogram of a urine sample of a fatal intoxication case. The urine sample was diluted with internal standard solution and water 1:10 prior to analysis. The indicated compounds are paracetamol (1), oxazepam (2), temazepam (3), mirtazapine (4),

diphenhydramine (5), methadone (6), and EDDP (7). In addition, nicotine and cotinine were detected. Diazepam- d_5 (I.S. 1) and doxepin- d_3 (I.S. 2) were used as internal standards

As this method uses ion spray ionization matrix effects, especially suppression of the analyte signals can occur and have to be taken into account.

The method was applied to serum and urine samples of forensic cases and was shown to be very useful as a complementary method to GC-MS screening, HPLC–diode-array detection (DAD), screening and immunoassays. An example is shown in Fig. 4. The urine sample was taken during an autopsy of a male person with suspected intoxication as the cause of death. The urine sample was diluted 1:10 with internal standard solution and water and injected into the LC-MS/MS system without further sample preparation or a hydrolysis step. The results fitted those obtained by immunoassay, and GC-MS screening additionally detected nordiazepam. It was detected by the MTS in MRM mode, but did not exceed the threshold of the IDA settings. Some signals in the chromatogram could not be identified after a library search of their corresponding EPI spectra. They might be caused by endogenous substances or compounds that are not included in our method.

Additionally, urine samples from other autopsies were analyzed by MTS. All the results were compared with those of a standardized GC-MS screening after a two-step liquid–liquid extraction and acid hydrolysis followed by liquid–liquid extraction and acetylation according to [1] (see Table 6).

Furthermore, the MTS was applied for serum samples of traffic offenses and a serum sample obtained during an autopsy after liquid–liquid extraction with chlorobutane at

pH 9. Identified compounds were quantified afterwards with validated LC-MS/MS methods as shown in Table 7.

Finally, two interlaboratory urine proficiency tests were performed by MTS and GC-MS screening and the results are compared in Tables 8 and 9.

Conclusion

The improved and automated MTS described in this paper is a powerful tool for routine analysis of serum and urine samples of intoxication cases for clinical and forensic purposes. Owing to the simplicity of the sample preparation especially for urine samples and the fast and automated processing of the data, results can be obtained within approximately half an hour, which is especially interesting for intoxication cases in order to select appropriate treatments.

In cases where only low volumes of samples are available, the dilution as a sample pretreatment offers the possibility to perform a screening analysis with volumes as low as 10 μ L of sample.

In general, semiquantitative analysis is possible with this method, but deuterated analogues have to be used as internal standards to compensate matrix effects. In principle, any kind of extraction can be combined with the LC-MS/MS screening procedure presented whenever the extraction is adequate for the analytes of interest, and the procedure can be applied to other matrices, such as oral fluid, hair, tissue samples, and gastric contents.

Table 6 Comparison of multi-target screening (MTS) and gas chromatography–mass spectrometry (GC-MS) results of autopsy cases. All urine samples were diluted 1:10 (v/v) prior to analysis by MTS.

Concentrations were determined with validated liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods. In all cases caffeine and nicotine or cotinine were identified with all methods

	Detected compounds	MTS dilution 1:10	GC-MS UNE	GC-MS UHyAC	LC-MS/MS (ng/mL)	
Autopsy case 1	Carbamazepine	+	+	–		
	Carbamazepine M	– ^a	+	+		
	Carbamazepine 10,11-epoxide	+	– ^a	– ^a		
	Metamizole M	– ^a	+	+		
	Metoprolol	+	–	–		
Autopsy case 2	Diphenhydramine	+	+	+		
	Diphenhydramine M	– ^a	+	–		
	Diazepam	–	–	+	66.4 (nordiazepam 68.2)	
	Oxazepam	+	–	+	171.2	
	Temazepam	+	–	–	22.7	
	Methadone	+	+	+		
	EDDP	+	–	–		
	Mirtazapine	+	–	+		
	Mirtazapine M	– ^a	–	+		
	Paracetamol	+	+	+		
	Paracetamol M	– ^a	–	+		
	Autopsy case 3	Methadone	+	+	+	
		EDDP	+	–	–	
Promethazine		+	–	+		
Promethazine M		– ^a	–	+		
Doxepin M		– ^a	+	–		
Diazepam		–	+	–	6.5	
Oxazepam		–	–	+		
Heroin		–	–	+ ^b		
Morphine 3-β-D-glucuronide		+	–	–		
Paracetamol		–	–	+		
Autopsy case 4	Doxepin	+	+	–	10,700	
	Doxepin M	– ^a	+	+		
	Methadone	+	+	+		
	EDDP	+	+	+		
	Oxazepam	+	–	+	392.4	

UNE standard extraction for urine, UHyAc extraction with acid hydrolysis and acetylation according to Maurer et al. [1], M metabolite(s), EDDP 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, + compound was identified, –compound could not be identified

^a Compound is not included in the method

^b Heroin was formed owing to the diacetylation of morphine.

A drawback of the method and the instrumentation is the exclusive use of positive ionization mode, because the polarity switching time of the mass spectrometer used would be too long for a combination with negative ionization mode. For the analysis of compounds that can only be ionized in negative mode, a separate method has to be used. This problem might be solved in the future by improved instrumentation. The GC-MS library of Maurer et al. used in this work, which has been developed over more than 20 years, has the advantage of containing many metabolites in addition to the drugs. The

inclusion of metabolites in an MTS procedure and the MS/MS library is another aim of future method development.

In general, the combination of different complementary methods such as immunoassays, HPLC-DAD, GC-MS, and LC-MS/MS (MTS) was shown to be a good approach for screening of urine and serum samples in forensic and clinical toxicology. Furthermore, the confirmation of a GC-MS screening result by LC-MS/MS screening fulfills the highest requirements for identification of compounds in cases with forensic consequences.

Table 7 Results of the analysis of serum/blood samples after liquid–liquid extraction with chlorobutane at pH 9. In each case 0.5 mL of serum/blood was extracted and reconstituted in 0.1 mL of solvent prior to analysis by MTS. The concentrations of the identified compounds were determined with validated LC-MS/MS methods

	Compounds detected by MTS extraction 5:1	Concentration (ng/mL)
Case 1 (traffic offense; serum)	Temazepam	83.1
	Nordiazepam	65.2
	Diazepam	1,000.8
	Mirtazapine	25.4
	Quetiapine	8.5
Case 2 (traffic offense; serum)	Nordiazepam	163.7
	Diazepam	85.6
	Quetiapine	157
Case 3 (traffic offense; serum)	Oxazepam	33.6
	Temazepam	847.5
	Zolpidem	321
Case 4 (traffic offense; serum)	Nordazepam	44.8
	Oxazepam	62.2
	Fentanyl	5.9
	Norfentanyl	3.5
	Metoprolol	NO
Case 5 (autopsy; femoral blood)	Tramadol	1,411
	Verapamil	416.4

NO not quantified

Table 8 MTS and GC-MS results of the analysis of an interlaboratory urine proficiency test. The spiked concentrations are shown in the *second column*. For MTS analysis the urine sample was diluted 1:10 (v/v)

Compound	Concentration (ng/mL)	MTS dilution 1:10	GC-MS UNE	GC-MS UHyAc
Amphetamine	100	–	–	–
Midazolam	350	+	+	+
Benzoylcegonine	900	+	–	–
Pentobarbital	1,200	– ^a	+	+
EDDP	750	+	–	+
Phencyclidine	400	+	+	+
Fentanyl	100	+	–	+
Fluoxetine	500	+	+	–

+compound was identified, –compound could not be identified

^a Compound is not included in the method

Table 9 MTS and GC-MS results of the analysis of an interlaboratory urine proficiency test. The spiked concentrations are shown in the *second column*. For MTS analysis the urine sample was diluted 1:10 (v/v) and additionally extracted with chlorobutane at pH 9

Compound	Concentration (ng/mL)	MTS dilution 1:10	MTS extraction 10:1	GC-MS UNE	GC-MS UHyAc
Codeine 6-glucuronide	150	– ^a	– ^a	–	– ^b
MDMA	500	+	+	–	+
Methylphenidate	800	+	+	+	–
Nortilidine	600	– ^a	– ^a	+	+
Nortriptyline	1,000	+	+	–	+
Zolpidem	600	+	+	+	+
LSD	30	–	+	–	–

+compound was identified, –compound could not be identified

^a Compound is not included in the method

^b Codeine was identified, and was formed by hydrolysis of codeine 6-glucuronide.

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