A Fast, Reliable and Automated Bench top LC-MS^n Drug Identification Solution for Clinical Research and Forensic Toxicology

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Introduction

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There is strong demand in both clinical research and forensic toxicology for automated, robust and sensitive analytical solutions to overcome the well-documented limitations of GC-MS, LC-UV and immunoassay technologies. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) combined with mass spectral library searching is an emerging strategy in this growing field of application. However, until now the adoption and successful implementation of this technology by non-MS experts has presented many difficult challenges. We herein describe the Toxtyper™, LC-Iontrap MS^n workflow: a robust and accurate solution for the rapid detection, identification and reporting of drugs in biological specimens with unprecedented ease of use. After sample extraction (urine or serum), a fast 11 minute-binary UHPLC separation is deployed prior to mass spectrometric analysis by the Toxtyper®. Drug and /or metabolite detection is confirmed by matching the generated MS, MS^2 and MS^3 spectra with retention time to the Toxtyper spectral library containing the corresponding information for 840 compounds of toxicological relevance. Fully automated data processing and evaluation is carried out and reports are directly sent to the user or the responsible toxicologist by email. We show how the Toxtyper™, workflow has been successfully tested by independent laboratories showing excellent inter-laboratory transferability of methods that generate accurate and reproducible results.

Experimental

Sample preparation

To demonstrate the interlaboratory transferability and reproducibility of the Toxtyper™ workflow, three spiked serum samples and one blank serum sample were sent to five different labs and analysed using seven separate Toxtyper™ LC-MS^n systems. Three mixtures of toxicologically relevant substances were spiked into blank human serum at different concentrations.
Additionally, a blank human serum sample was extracted. Sample preparation was carried out using the following liquid-liquid extraction (LLE) protocol. Serum (1 mL) was spiked with 50 ng of D5-diazepam as an internal standard and then mixed with 0.5 mL borate buffer (pH 9) and 1.5 mL 1-chlorobutane. After a 3 min mixing step, the solution was centrifuged at 4000 × g for 5 min. The organic phase was separated, aliquoted, and evaporated at 40°C with N_2. These aliquots were forwarded to the 5 participating labs, where the residues were redissolved in 25 µL eluent A/B (50:50; v/v;)

LC Conditions	
LC system	Thermo Dionex Ultimate3000 RSLC
Eluent A	H_2O, 0.1% formic acid, 2 mM ammonium formiate, 1% acetonitrile
Eluent B	Acetonitrile, 0.1% formic acid, 2 mM ammonium formiate, 1% H_2O
Analytical column	Acclaim® RSLC 120 C18 2.2 µm, 120A, 2.1 x 100 mm
Flow rate	500 µl/min
Gradient:	0.0 to 1.0 min: 1% B
	1.0 to 8.0 min: 1% B to 95% B, linear
	8.0 to 9.0 min: 95% B
	9.0 to 9.06 min: 95% B to 1% B, linear
	9.06 to 11 min: 1% B
MS Conditions	
Mass Spectrometer	Toxtyper™ AmaZon Speed MS^n system
Scan mode	UltraScan 32.500 *m/z* sec^{-1}
Scan range	70 - 800 *m/z*
Source	Electrospray ionisation (ESI)
Polarity	Zero Delay Alternating +/- polarity switching
MS^n Acquisition	Data dependent MS^2 and MS^3 with Scheduled Precursor List of 830 cpds
	Active exclusion after 1 spectrum, reconsider if intensity increase by factor 5

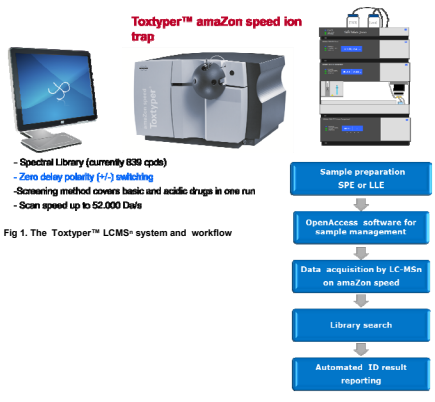

Fig 1. The Toxtyper™ LCMS^n system and workflow

The toxicological compounds of interest in this interlaboratory study were chosen without consideration to their retention times or molecular masses and were spiked into blank human serum at different concentrations as detailed in Table 1.

Sample 1	Sample 2	Sample 3
Methadone (250)	Trimipramine (100)	Duloxetine (600)
EDDP (50)	Amitryptiline (100)	Nordoxepin (300)
Diazepam (100)	Zolpidem (500)	Mirtazapine (50)
Nordazepam (500)	Midazolam (150)	Metoprolol (200)
Oxazepam (200)	α-OH-midazolam (50)	
Temazepam (100)	Fentanyl (3)	
	Lidocaine (200)	

Table 1. Compounds spiked in human blank serum to test the lab to lab transferability of the identification workflow.

LC-MS^n conditions

Two microliters of the re-constituted sample was injected onto an Ultimate3000 RSLC system using seven different amaZon speed ion trap systems. Each system was used for the generation of MS and MSn spectra with continuous polarity switching. Data was acquired using a data-dependent scheduled precursor list (i.e. an inclusion list for 830 target compounds).

Data Acquisition and Spectral Library Searching

The Toxtyper™ MS^n spectral library was developed in close collaboration with the Forensic Institute in Freiburg (University Medical Center, Germany[2]) and included spectral information and retention time data for 830 compounds of forensic and clinical research importance.
All samples in the interlaboratory test were processed using Data Analysis 4.1 in a completely automated manner using Compass OpenAccess. Compass OpenAccess is a 'walk up and go' graphical user interface that allows complete novice users to execute the Toxtyper workflow in a routing environment (see fig2). After completion of a run, the user received a PDF report of the LC-MS^n results; either by logging onto the web based COA system or by email to a Smartphone. The automatically generated reports from the different labs were evaluated to demonstrate transferability of the Toxtyper solution and compare the overall performance of the different LC-MS ion trap systems. If a substance was not identified, the respective raw data file was inspected manually to find the cause.

Compass OpenAccess: 'WALK UP AND GO' USER INTERFACE

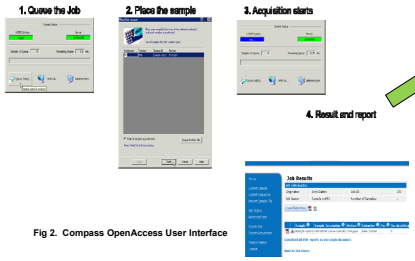

Fig 2. Compass OpenAccess User Interface

Trimipramine was not identified by one lab due to extensive coelution of matrix giving a mixed MS^2 spectrum yielding a low spectra purity score value below the cutoff for reporting. Metoprolol from sample 3 was not identified by two systems at HUG1 and HUG2. This was due to coelution with mirtazapine, which led to a mixed MS^2 spectrum and subsequently to a score value below the cut-off for ID reporting.
It should be noted that metoprolol and mirtazapine not only have very similar retention times, but also differ only slightly in mass (2 Da). Common false positives were identified in the blank serum sample and the other samples, but these could be easily excluded after manual inspection of the reports and the respective raw data files. For example, a common false positive was benzododecinium. This compound is used as skin disinfectant during blood withdrawal and is present in the sample as a contaminant. Figure 4 shows the MS^2 spectra of amitriptyline recorded from spiked serum extracts of all participants and the respective library spectrum. SmartFrag technology provides reproducible fragmentation results that lead to the highest level of lab-to-lab transferability and reproducibility.

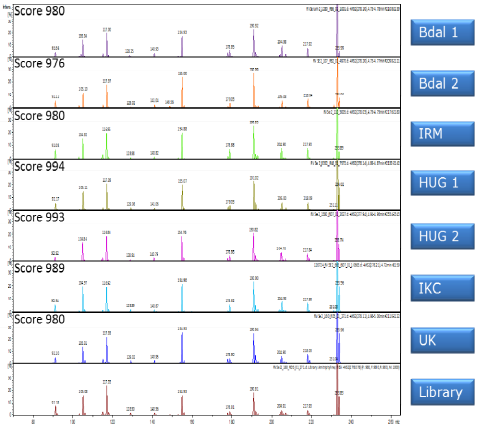

Fig 3. SmartFrag™ generates reproducible MS^2 spectra from 7 Toxtyper systems

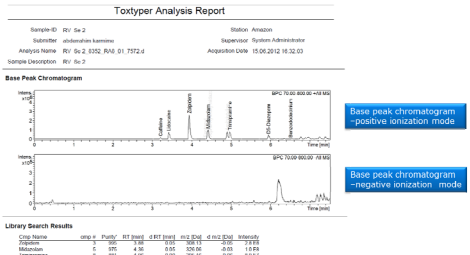

Fig 4. Example of automatic Toxtyper ™report produced from Compass OpenAccess

Results and Discussion

All compounds spiked into sample 1 could be identified by all participating labs wit han analysis time of approximately 11 minutes per sample. Several substances, for example, diazepam, and temazepam in sample 1, were present at low concentrations, demonstrating the high sensitivity of the procedure. The results are summarized in Table 2.

Spiked Compounds / Sample 1	Participants IKC	IRM	HUG 1	HUG 2	UK	BDal 1	BDal 2
Methadone	✓	✓	✓	✓	✓	✓	✓
EDDP	✓	✓	✓	✓	✓	✓	✓
Diazepam	✓	✓	✓	✓	✓	✓	✓
Nordazepam	✓	✓	✓	✓	✓	✓	✓
Oxazepam	✓	✓	✓	✓	✓	✓	✓
Temazepam	✓	✓	✓	✓	✓	✓	✓
Sample 2	**IKC**	**IRM**	**HUG 1**	**HUG 2**	**UK**	**BDal 1**	**BDal 2**
Amitriptyline	✓	✓	✓	✓	✓	✓	✓
α-OH-midazolam	✓	✓	✓	✓	✓	✓	✓
Fentanyl	✓	✓	✓	✓	✓	✓	✓
Lidocaine	✓	✓	✓	✓	✓	✓	✓
Midazolam	✓	✓	✓	✓	✓	✓	✓
Trimipramine	✓	✓	✓	✓	⊙	✓	✓
Zolpidem	✓	✓	✓	✓	✓	✓	✓
D5-diazepam (IS)	✓	✓	✓	✓	✓	✓	✓
Ingredient of Serum							
Caffeine	✓	✓	✓	✓	✓	✓	✓
Theobromine	-	✓	-	✓	-	-	✓
Sample 3	**IKC**	**IRM**	**HUG 1**	**HUG 2**	**UK**	**BDal 1**	**BDal 2**
Duloxetin	✓	✓	✓	✓	✓	✓	✓
Metoprolol	✓	✓	⊙	⊙	✓	✓	✓
Mirtazepine	✓	✓	✓	✓	✓	✓	✓
Nordoxepine	✓	✓	✓	✓	✓	✓	✓
D5-diazepam (IS)	✓	✓	✓	✓	✓	✓	✓
Ingredient of Serum							
Caffeine	✓	✓	✓	✓	✓	✓	✓
Theobromine	-	✓	-	-	-	-	-

Table 2: Results from the interlaboratory test

Conclusion

The Toxtyper™ workflow is a fast and robust tool for the identification and confirmation of xenobiotics in clinical research and forensic toxicology. The combination of MS^n spectral information with retention time meets common criteria for identification of analytes. The results of the inter-laboratory test demonstrated the efficiency and transferability of the complete workflow over seven independent systems within different laboratories. The unrivalled degree of automation offered by Compass OpenAccess from sample submission, through to data analysis and reporting, makes the Toxtyper™ ideal for use in environments, where a high degree of operator skill may not always be available. The turnkey deployment of the workflow coupled with high detection rates from all laboratories demonstrates the superior performance of the Toxtyper™ over immunoassay, GC/MS and LC-triple quad systems for toxicological drug identification.

The use of additional commercial libraries to extend the scope of this technology towards "General Unknown Screening" workflows is currently being investigated.