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Are they among us? Screening for drugs of abuse and new psychoactive substances in pooled human urine and wastewater samples

Marie Mardal[a,c] and Markus R. Meyer[b,c]
[a]Department of Forensic Medicine, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark
[b]Department of Pharmacology and Pharmacoepidemiology, Heidelberg University Hospital, Heidelberg, Germany.
E-mail: Markus.Meyer@med.uni-heidelberg.de
[c]Department of Experimental and Clinical Toxicology, Saarland University, Homburg(Saar), Germany

Introduction

New psychoactive substances (NPSs), also known as "legal highs", are of growing concern to drug legislators and police authorities. Although NPSs are sold by internet vendors and local head shops as "research chemicals not for human consumption", they are frequently consumed to obtain psychotropic effects. When entering the international drug markets, only a little information is available about their safety profiles and it is usually up to research institutions to perform basic toxicological studies post-marketing. As an NPS should mimic the effect of a traditional drug of abuse (DOA) such as cocaine, amphetamine and ecstasy (MDMA), chemical modifications to circumvent legislation but retaining the psychotropic effect are introduced into the molecules. Examples of such designed NPSs are shown in Figure 1. NPSs might also be previous drug candidates from pharmaceutical companies or research facilities, such as most of the synthetic cannabinoids (e.g. WIN55, 212-2 and JWH-018), benzodiazepine analogues and the so-called NBOMe.

In Europe, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) monitors which NPSs enter the market through their early warning system. Information is collected locally from reports of seized materials, monitoring of online forums, internet vendors and head shops. Information on consumption of DOA and NPSs is collected, amongst others, from comprehensive toxicological screening methods performed from clinical, forensic, driving-under-the-influence-of-drugs and work-place biological samples. For such comprehensive toxicological screening procedures, urine and blood are the preferred biological samples. However, hair, oral fluid, breath and nail analysis each have their special place in drug screening.[1] It can be challenging to obtain biological samples for non-routine purposes, including for epidemiological research, as ethical approval would be required.

Therefore, analysis of pooled biological samples from a larger group of people might be an alternative as it does not usually require such ethical approval. However, it is important to emphasise that ethical aspects and implications should always be considered before initiating experiments with materials of human origin. Pooled urine analysis (PUA) can provide a local-based snap-shot of which DOA and NPSs are consumed in the sampled population. One of the first studies ever was performed in the city of London, by analysing samples from 12 urinals for DOA and NPSs over a six-month period. This revealed consumption of various NPSs, such as 5-APB and mephedrone.[2] Another source of pooled urine, and also pooled faeces, is wastewater (Figure 2). In contrast to the pooled urine collected via urinals, wastewater samples are heavily diluted with industrial wastewater and run-off water, which requires very sensitive analytical strategies. In wastewater-based epidemiology (WBE), drug consumption trends are analysed by quantitation of DOA and/or NPSs in representative wastewater samples and correlated with the number of people served by the wastewater treatment plant.[3]

Wastewater-based epidemiology

Quantitation of particular analytical targets of DOA and/or NPSs in wastewater samples can reveal time-dependent changes in drug consumption patterns and provide estimations on the amount of consumed drugs per 1000 inhabitants per day.[3] These data can then be compared amongst cities, to obtain spatial resolution on drug consumption patterns. European research institutions

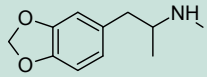
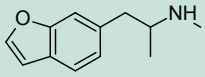
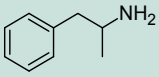
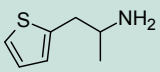
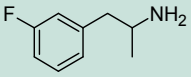
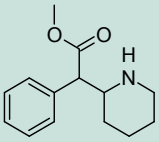
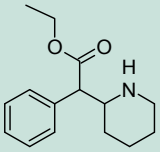
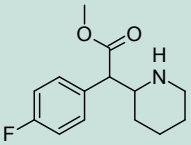
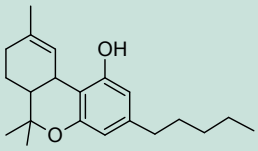
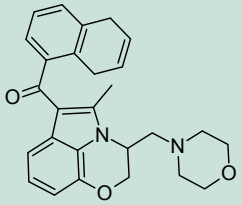
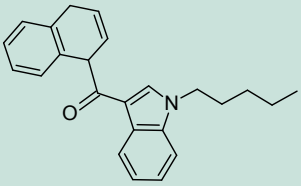
Traditional psychoactive substances	Corresponding new psychoactive analogues	
		
MDMA (Ecstasy)	6-MAPB (Benzofury)	
		
Amphetamine	Methiopropamine	2-Fluoroamphetamine
		
Methylphenidate (Ritalin®)	Ethylphenidate	4-Fluoromethylphenidate
		
THC	WIN55,212-2	JWH-018 (Spice)

Figure 1. Conventional drugs of abuse and new psychoactive substances (street names in brackets) mimicking their effect. Bioisosteric substitutions of the MDMA methylenedioxy ring by a furane moiety in 6-MAPB, modification of the length of the alkyl chains in ethylphenidate, substitution of hydrogens with halogens in 4-fluoromethylphenidate and 2-fluoroamphetamine.

participate in a yearly monitoring campaign analysing DOA in wastewater in their respective cities. The results are frequently published by EMCDDA as a part of their yearly drug report (http://www.emcdda.europa.eu/edr2016).

One important task before WBE can be applied is to identify the main human excretion products, which may serve as analytical targets in the wastewater samples. Such data from controlled administration studies are available for some of the most common DOA but not for NPSs. Identified analytical targets have also to be tested for their stability during the transit time in the sewer networks.[4] Unfortunately, particularly metabolites of NPSs are often not known and commercially available. For this reason, the unchanged drug is commonly used as the analytical target, although this does not allow distinction between drug discharge or consumption. One way to identify main human urinary excretion products when controlled administration studies are not available is to screen authentic human urine samples from individuals having consumed the compounds of interest. This is often performed in forensic and clinical labs, where urine samples are available. Another option is PUA using urine samples collected at events where people are assumed to consume DOA and NPSs. By applying comprehensive standard urine screening protocols and updated screening libraries, it should be possible to identify main and possibly new metabolites for DOA and NPSs.

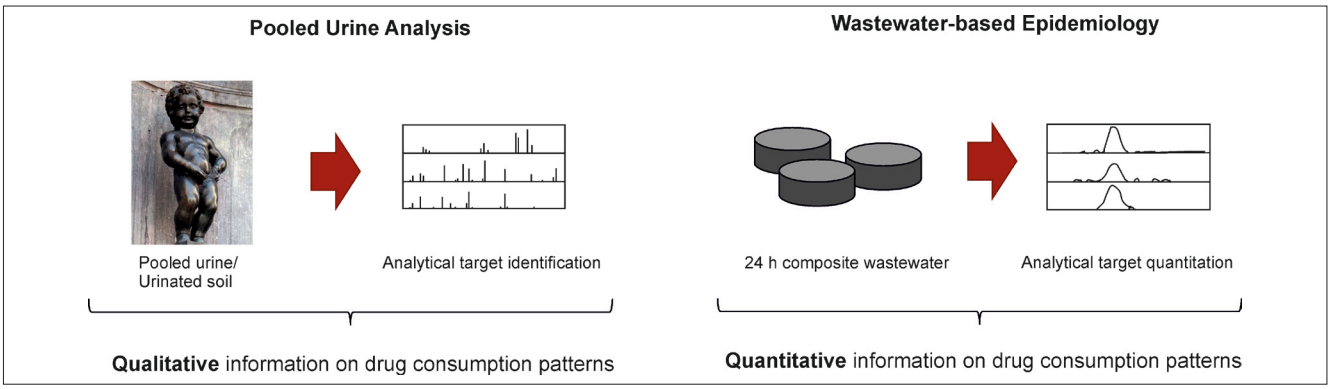


Figure 2. Type of samples collected and screening method applied for pooled urine analysis and wastewater-based epidemiology.

Information from urine screening studies can then help in identifying analytical targets for WBE.

Analysis of urinary samples collected at music festivals

For the study by Mardal *et al.*,[4] pooled urine samples were collected from the camping area of a Scandinavian festival in 2014 with the purpose of performing PUA. Samples were subsequently screened to identify consumed DOA and NPSs, and to estimate which analytical targets could be suitable for WBE. Three types of samples were collected:

1) Pooled urine using a Peetree urinal put on a wall, which was connected via a plastic hose to a container located in a protected environment. When the plastic container had more than 3.5 L (defined as a minimum level of 10 contributing urinations), a subsample was extracted, an internal standard added and the remaining urine was discarded into an adjacent toilet.
2) Pooled urine extracted from tulip urinals using a syringe and a plastic hose and addition of internal standard.
3) Grab samples of urinated soil collected from areas where urine accumulated on the ground (fences and between bushes). Five examples of urinated soil samples are presented in Figure 3.

Different advantages and disadvantages were associated with the chosen collection methods. Method 1 was labour-intensive and time-consuming, but it was possible to approximate the amount of urinations per sample and less analytical carry-over should be expected than for method 2. The clear advantage of method 2 was ease of sampling, and having a larger contributing population, and generally, more DOA were identified in urine samples collected using method 2. Method 1 and 2 have a gender bias, as only men would use these urinal types, whereas method 3 could capture urine from men and women. However, limited information was available on stability and sorption of the analytical targets of interest on the soil, urinal and container walls.

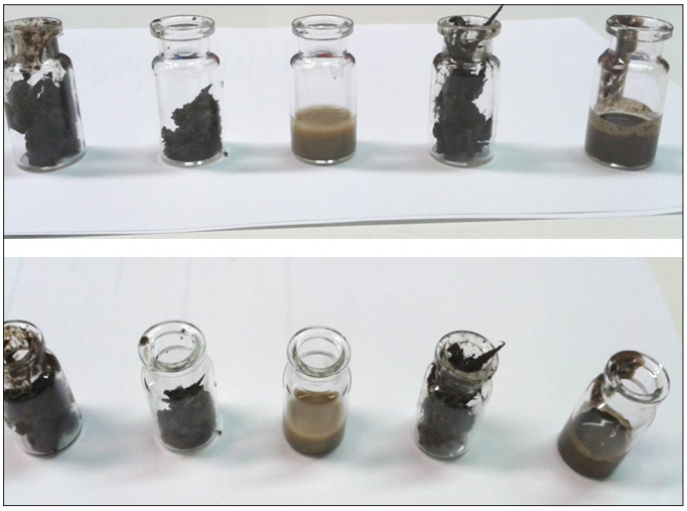


Figure 3. Examples of urinated soil samples collected using method 3.

Also the soil residence time could not be estimated.

Screening of biological samples for therapeutic drugs, DOA and their metabolites

Analytical target concentration levels of therapeutic drugs, DOA and NPSs, are low in pooled biological samples compared to biological samples from an individual drug user. In order to detect these lower concentration levels, sophisticated sample work-up prior to analysis, and high sensitivity, selectivity and reproducibility of the analytical methods are required. The wastewater samples used in WBE are usually concentrated by a factor of 100–500 in volume prior to analysis by solid phase extraction whilst PU can be prepared using standard urine screening approaches established in forensic and clinical labs (see below).

Chromatographic separation prior to detection is performed by liquid or gas chromatography. Gas chromatography-mass spectrometry (GC-MS) is a powerful comprehensive screening tool, when mass spectral databases are available for compound identification. However, samples need derivatisation prior to analysis to make compounds with polar groups volatile. Ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) is the method of choice for quantification of analytical targets at low concentration levels, but also for targeted screening purposes. Reversed phase liquid chromatography (RPLC) columns are standard in LC-MS analysis of (pooled) biosamples, whereas the hydrophilic interaction liquid chromatography (HILIC) columns can be advantageous for certain molecules too polar for retention on conventional RPLC columns. Urinary metabolites of DOA usually are more hydrophilic than their parent compounds and certain hydrophilic metabolites, including phase II metabolites and amphetamine-like compounds showed improved retention on HILIC columns.[4–6] Finally, chiral LC columns are used in WBE, separating the enantiomers of analytical targets of DOA to identify if the enantiomeric ratio is likely to originate from consumption rather than discharged drug.[7] Nano-LC has not yet been applied to wastewater, but recent application to human urine samples collected from NPS users, revealed increased MS signal intensities using nano-LC-(nano-ESI)-MS/MS when compared to UHPLC-(ESI)-MS/MS.[8]

Mass spectrometric analysis of pooled biological samples can roughly be divided into three categories: targeted methods for quantifying a predefined list of targets, data-dependent acquisition and data-independent acquisition.

Targeted methods are used when sensitivity is needed and when analytes of interest are identified beforehand. High resolution (HR) instruments are equally suited for such quantifications

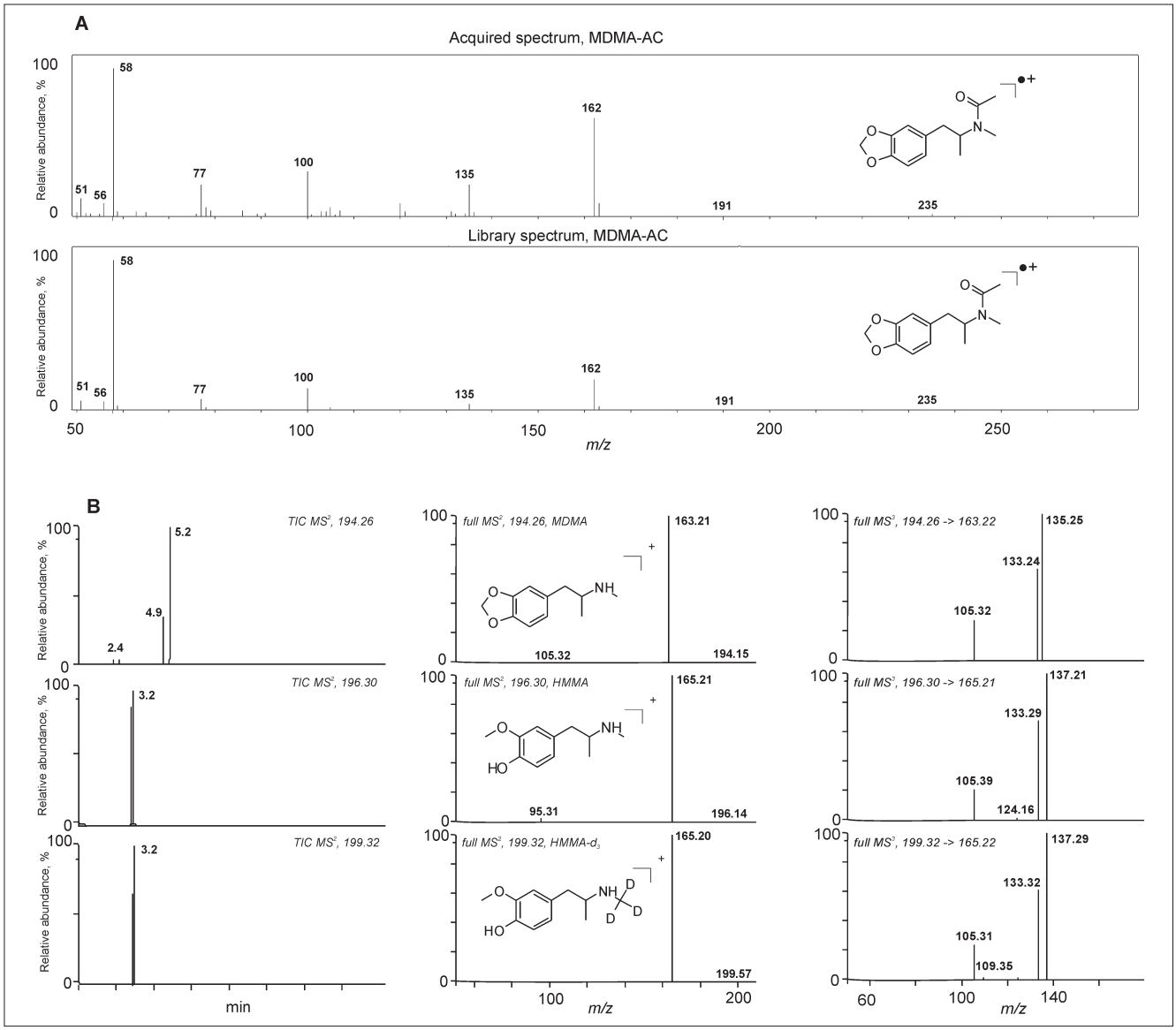


Figure 4. Mass spectral identification of MDMA by data-independent acquisition (A) and data-dependent acquisition (B) in pooled urine samples. A: GC-EI$^+$-MS of acetylated pooled urine extract, with acquired experimental background-subtracted spectrum (top) and reference library (H.H. Maurer, K. Pfleger and A.A. Weber, *Mass Spectral and GC Data of Drugs, Poisons, Pesticides, Pollutants and their Metabolites*", 5th Edn, 2016) spectrum (bottom). B: UHPLC-ESI$^+$-MS^n (ion trap) acquired data from conjugate cleaved pooled urine showing MDMA, its main phase I metabolite HMMA and the deuterated internal standard HMMA-d3 with total ion chromatogram (TIC) of the acquired MS^n scans with specified precursor ion, full MS2 and full MS3 of the most abundant fragment ion in MS2.

as triple quadrupole instruments.[9] In data-dependent acquisition mode a survey scan is performed, after which a pre-defined number of MS^n spectra are acquired by isolating the most abundant ions in a defined *m/z* range. Analytical targets can then be confirmed by comparing acquired data with database entries.[10] This method provides clean MS^2 spectra, however, if analytes of interest are co-eluting with other compounds of higher intensity, second injections are required for structural identification. Data-independent acquisitions are routinely employed for drug screening using both LC- and GC-MS. For LC-HRMS, an un-biased all-ion fragmentation method is possible, where data is collected using high and low collision energies. Data from the different collision energies are associated post-analysis by deconvolution algorithms. A great advantage of HR data-independent acquisition is the option of retrospective data-analysis.

Standard urine screening approach applied to pooled urine

When performing screening of pooled urine, a comprehensive analysis protocol must be applied to cover all groups of NPSs. The in-house standard urine screening protocol applied to the pooled urine and urinated soil collected at the Scandinavian festival involved sample pretreatment either with or without enzymatic conjugate cleavage, sample prep-

aration by precipitation and solid phase extraction on C18 or HCX (mixed-mode non-polar and strong cation exchange retention) cartridges. The extracts were analysed in data-dependent mode by LC-MS^n (TF LXQ, linear ion trap), LC-HR-MS/MS (TF Q-Exactive, Orbitrap), and in data-independent mode by GC-MS analysis, the latter using acetylated extracts. Data from each instrument was evaluated with libraries containing DOA, NPSs and their metabolites.[11] This approach allowed the detection of analytical targets with different physiochemical properties and different detection limits on the used instruments. Also the identification confidence was increased by analysing multiple analytical targets on complementary instrumentation. Several therapeutic drugs were identified in the pooled urine and urinated soil samples and the main detected DOA were cocaine, MDMA (see, for example, Figure 4), MDEA, MDA (most likely as metabolite from MDMA) and ketamine. No NPSs were identified, which might be explained by absence of consumption, too low concentration levels or because the analytical targets were not contained in the applied screening databases.

Future perspectives for NPS monitoring

In order to detect consumed NPS in pooled biological samples, there are three areas which should be improved.

1) Improvement of methodologies for analytical target identification of NPSs. *In vitro* systems such as hepatocytes or liver cell preparations for fast metabolite identification of NPSs are gaining increased attention, as they can produce both phase I and II metabolites but in low concentration.[5] Publication of results is mainly achieved by peer-reviewed publications. Unfortunately, the peer-reviewing process often leads to a long time span between NPS identification on the international drug market and publication of the analytical targets and reference databases.
2) A greater sensitivity and/or selectivity of the analytical methods should help to quantify less frequently consumed compounds. Increased selectivity could be achieved through adding an additional separation mechanism between the LC and MS system such as ion mobility. Implementation of nano-LC combined with nano-ESI MS could further help to increase sensitivity.
3) Mass spectral databases for NPSs and their analytical targets should continuously be updated to cover the expanding types of NPS and their metabolites, preferably as soon as they are detected on the drug markets. Certain NPSs, including synthetic cannabinoids and benzodiazepines, are mainly excreted as their metabolites. A data-mining approach obtaining increased attention in environmental sciences for emerging pollutants is the non-targeted screening method, where sophisticated post-processing tools are employed to reveal hidden patterns in three-dimensional mass spectral data corresponding to molecules not present in target screening databases. Such hidden mass spectral information in pooled biological samples could potentially be NPSs or metabolites. Non-targeted screening is currently not suitable for routine screening of biological samples. However, it is a helpful tool in special cases, where conventional screening methods fail to provide data explaining symptoms or autopsy findings from a patient or deceased suggesting drug consumption or intoxication.[12]

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