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Rapid sample pre-treatment prior to GC–MS and GC–MS/MS urinary toxicological screening

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ABSTRACT

Drug screening is an important issue in clinical and forensic toxicology. Gas chromatography coupled to mass spectrometry (GC–MS) remains the gold standard technique for the screening of unknown compounds in urine samples. However, this technique requires substantial sample preparation, which is time consuming. Moreover, some common drugs such as cannabis cannot be easily detected in urine using general procedures. In this work, a sample preparation protocol for treating 200 μL of urine in less than 30 min is described. The enzymatic hydrolysis of glucuro-conjugates was performed in 5 min thanks to the use of microwaves. The use of a deconvolution software allowed reducing the GC–MS run to 10 min, without impairing the quality of the compound identifications. Comparing the results from 139 authentic urine samples to those obtained using the current routine analysis indicated this method performed well. Moreover, additional 5-min GC–MS/MS programs are described, enabling a very sensitive target screening of 54 drugs, including THC–COOH or buprenorphine, without further sample preparation. These methods appeared as an interesting alternative to immuno-assays based screening. The analytical strategy presented in this article proved to be a promising approach for systematic toxicological analysis (STA) of drugs in urine.

1. Introduction

Drug screening is an important issue in clinical and forensic toxicology that provides essential information for both making diagnosis and progressing toward confirmatory and quantitative analyses. Systematic toxicological analysis (STA), should ideally enable the detection and identification of any untargeted substance of toxicological interest in biological fluids. This is not an easy task as the detection capability strongly depends on the matrix, sample preparation, analysis technique, and compound database used. In this way, a STA generally consists of a combination of analytical procedures, primarily immunoassays, chromatography and mass spectrometry, which give a precise overview of the substances that were taken by comparing the results [1].

Gas chromatography coupled to mass spectrometry (GC–MS) is a very powerful tool for toxicological screening in STA procedures, as it combines good separation with reproducible mass fragmentation. Electronic impact (EI) fragmentation allows for the construction of suitable inter-instrument compound libraries, which greatly facilitates identifying the detected compounds [2] through direct comparison to homemade or commercial reference libraries. Due to its easy implementation, GC–MS has become the gold standard technique for screenings in toxicological laboratories [3]. However, identification can fail if the mass spectrum of the substance is contaminated with peaks caused by insufficient chromatographic separation, column bleeding or some other source [4]. Several groups have worked on finding means to improve the identification performances of GC–MS [5–9]. In this way, a freeware deconvolution software named AMDIS (Automated Mass Spectral Deconvolution and Identification Software; http://chemdata.nist.gov/mass-spc/amdis/) was developed to allow both the deconvolution of complex spectra and the matching of a purified spectrum to a reference spectrum [4]. The use of this type of algorithm to reduce the influence of chromatographic resolution on the identification capability is very helpful, and several publications have demonstrated its suitability for forensic applications [10,11].

Even when using the most sophisticated algorithm, identification can still fail if the acquired spectrum quality is low. This may occur for analytes that do not respond well to conventional GC–MS procedures or have concentrations that are too low for the analytical system. In this case, tandem mass spectrometry may be an interesting alternative and very sensible tool for drug screening. GC–MS/MS is more commonly used for quantitation purposes, but it is perfectly compatible with drug identification [12] and was successfully used in environmental investigations [13] or to screen doping substances in human urine [14]. To the best of our knowledge, no STA application was described, which is surprising.

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Urine is generally used as matrix of choice for drug screening, as this matrix concentrates a wide range of drugs and/or metabolites of interest. Urine is also very convenient for screening because large amounts can be easily and non-invasively collected [15]. The main limitation when dealing with GC–MS procedures is that substantial sample preparation is required to make urine compatible with GC relative to various "dilute-and-shoot" procedures described for LC–MS analysis [16,17]. The first step is generally hydrolysis, as many drugs are excreted in urine as acetalic and acylalic glucuronides or sulfuric esters [18]. It is necessary to convert these conjugates into more easily extractable compounds to perform the GC separation. Hydrolysis can be rapidly achieved by refluxing in concentrated hydrochloric acid; however, this acidic cleavage is aggressive and can lead to both a relatively unclean hydrolysate and possible compound loss [19], which restraints its application [20]. Several authors prefer enzymatic hydrolysis using β-glucuronidase and arylsulfatase [11,20–24], but enzymatic hydrolysis requires several hours to be effective. However, it has been shown that enzymatic hydrolysis reactions and their derivatization can be accelerated by microwave irradiation [25–27] or ultrasonification [24], which can greatly reduce the time required to perform GC–MS screening. The analytes from the hydrolyzed urine must then be extracted, as GC is not compatible with aqueous solutions. This can be achieved through liquid–liquid extraction (LLE) or solid-phase extraction (SPE). The solvent properties, pH and, for SPE, stationary phase strongly effect the drug recoveries based on their physico–chemical properties. Therefore, separate extraction procedures are generally proposed for different classes of compounds, such as acidic and neutral drugs or basic drugs [2,19]. LLE is the traditional extraction method for drug screening because it is a cheap, simple and effective procedure for a wide range of analytes [21,28,29]. Many solvents or solvent mixtures have been proposed with the goal of enlarging the polarity range of the extracted substances without increasing the background noise. Therefore, many authors have described the use of apolar solvents such as ethyl acetate, dichloromethane and n-butyl chloride [30] containing varying proportions of miscible polar solvents such as acetone or isopropanol [2,27]. The use of SPE has also been widely studied, especially in publications focusing on automated sample treatment [21,27]. However, SPE is more exclusive, expensive and time consuming than LLE and usually generates more waste in term of solvents and consumables. For both LLE and SPE techniques, the actual trend is to miniaturize the extraction system to simplify the manipulations and minimize the solvent volumes and time required to perform the extraction [29]. Finally, the derivation of compounds with polar moieties is also required to enhance their volatility and thermal stability [31].

The purpose of this work was to combine the benefits of recent developments in sample preparation and data handling to develop a rapid and reliable urinary screening procedure. The objective was to limit the sample preparation time to less than 30 min, including hydrolysis, and the GC–MS analysis time to 10 min. The use of tandem mass spectrometry (GC–MS/MS) as a complementary analysis method was also investigated to show that it is an effective tool for extending the detection capabilities of GC-based STA procedures.

2. Experimental

2.1. Chemicals and reagents

All reference compounds and deuterated analogs were purchased at 1000 μg/mL or 100 μg/mL in methanol or another suitable solvent from Cerilliant (Round Rock, USA) or Lipomed (Arlesheim, Switzerland). Working standard mixtures were prepared by diluting these stock solutions in methanol to obtain the concentration of interest. Trimipramine-d3 at 10 μg/mL in methanol was prepared separately as an internal standard (IS). After use, both the stock and working solutions were stored at −20 °C.

Acetic anhydride and pyridine were obtained from Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany), respectively. High-purity grade methanol, dichloromethane (CH_2Cl_2) and hexane were obtained from Merck, n-butyl chloride (BuCl) was obtained from Romil (Rüti bei Büren, Switzerland), and both isopropanol (isoPrOH) and ethyl acetate (EA) were obtained from Sigma Aldrich (St Louis, USA). Enzymatic hydrolysis was performed using glusulase II, which contained both β-glucuronidase and β-glucuronide sulfatase, from Perkin Elmer (Waltham, USA). Borate buffer was prepared from boric acid from Fluka, and both potassium chloride and sodium carbonate from Merck. A pH of 9 was obtained by adjusting with an aqueous solution of sodium hydroxide from Merck. The immunoassay calibrators used for cannabinoids and buprenorphine were Syva® EMIT® calibrator/control Level 3 from Siemens Healthcare Diagnostics (Tarrytown, USA) and CEDIA® Buprenorphine 5 ng/mL calibrator from Thermo Fisher Scientific (Fremont, USA), respectively. All human urine was supplied by the University Center of Legal Medicine (Geneva, Switzerland).

2.2. Preparation of spiked urines

Urine was spiked to the concentrations of interest for all tested compounds by adding a suitable volume of the corresponding stock solution to a plastic microtube. The solvent was then evaporated to dryness under a gentle stream of nitrogen, and urine was added to the residue. The spiked urine was strongly vortexed to ensure the dissolution of the analytes.

2.3. Sample preparation

After spiking with 5 μL of the IS, 200 μL of urine were hydrolyzed in a 2 mL plastic microtube with a locking cap using 20 μL of glusulase II under microwave irradiation (350 W) for 5 minutes (domestic microwave oven, Whirlpool FT331/1). The hydrolysate was brought to a pH of 9 using 200 μL of a borate buffer. The LLE was performed using 1 mL of n-chlorobutane/isopropanol (4:1, v/v) with strong horizontal shaking (20 moves/s) for 2 min. After centrifuging for 1 min at 21,100 g, the upper organic phase was transferred into a 1.5 mL plastic microtube and evaporated to dryness under a gentle stream of nitrogen. Derivatization of compounds containing polar moieties was achieved by adding 20 μL of acetic anhydride and 20 μL of pyridine to the extracted residue. After mixing, the microtubes were again irradiated for 5 min at 160 W and finally evaporated to dryness under nitrogen. The residue was reconstituted using 50 μL of methanol, and 1 μL was injected into the analytical system.

2.4. Microwave hydrolysis evaluation

To evaluate the efficiency of microwave irradiation for enhancing the enzymatic hydrolysis, eight urine samples containing buprenorphine, oxazepam, temazepam, morphine and/or codeine were hydrolyzed in triplicate ($n=3$) both as previously described and following the routine method, i.e., 3 h of heating at 60 °C with 20 μL glusulase II. After hydrolysis, the extraction and derivatization procedures remained as described above. The extracts were analyzed using both MS and the corresponding MS–MS methods. The hydrolysis yield was evaluated by comparing the obtained peak areas after microwave irradiation to those obtained after 3 h

heating, which were taken as a reference. The concentration of each analyte was evaluated by comparing the ratio between the analyte and the IS peak areas, both before and after hydrolysis, to those of extracts from blank urine spiked with the compounds cited above at concentrations of 50 and 500 ng/mL.

2.5. LLE evaluation

The extraction procedure was evaluated by extracting urine samples spiked with amphetamine, nordiazepam, bromazepam, α-hydroxymidazolam, morphine, codeine, methadone, zolpidem, venlafaxine, methylecgonine, phenobarbital, 11-nor-9-carboxy-Δ_9-tetrahydrocannabinol (THC–COOH) and clozapine at a concentration of 500 ng/mL following the procedure described above. These extracts were injected into the analytical system, analyzed using the corresponding MS–MS method, and compared to neat solutions of the selected compounds in MeOH at a corresponding concentration. These extractions were performed in triplicate ($n=3$).

Extraction recoveries were also evaluated using BuCl and two solvent mixtures found in the literature, i.e., CH_2Cl_2:hexane:EA (5:4:1, v/v/v) and EA:isoPrOH:CH_2Cl_2 (3:1:1, v/v/v) [17]. The sample preparations remained as described above, except for the extraction solvent mixture.

2.6. Equipment

All analyses were performed on a Varian CP 3800 gas chromatograph (Walnut Creek, CA, USA) equipped with a Varian CP-8400 autosampler and Varian 300-MS triple quadrupole mass spectrometer (Walnut Creek, CA, USA). Both data acquisition and analysis were performed using Varian MS Workstation software (Version 6.9.3).

2.7. Chromatographic conditions

All substances were separated on a ZB-5MS fused-silica capillary column (15 m × 0.25 mm i.d., film thickness 0.25 μm with 10 m of inert guard column) from Phenomenex (Torrance, USA) and high-purity helium 50 (99.999%) was used as the carrier gas with a constant flow of 1 mL/min. The injector temperature was set to 250 °C, and splitless mode injection was performed.

For the GC–MS analysis, the initial oven temperature was held at 85 °C for 0.5 min, increased to 145 °C at 30 °C/min and then to 285 at 70 °C/min. The final temperature was held for 5.5 min for an analysis time of 10 min.

The temperature program for the GC–MS/MS analyses was adapted to the targeted analytes. Five methods, which correspond to the five analyte groups, have been developed. These methods are described in Table 1.

Table 1
GC programs corresponding to the 5 compound groups. The initial temperature is labeled as T0, intermediate temperature as T1 and final temperature as T2.

Group	T0 (°C)	Hold time (min)	Gradient 1 (°C/min)	T1 (°C)	Hold time (min)	Gradient 2 (°C/min)	T2 (°C)	Hold time (min)	Total time (min)
A and E	85	0.10	–	–	–	70	285	2.04	5.00
B	150	0.10	–	–	–	70	325	2.40	5.00
C	60	0.10	30	120	0.00	70	285	0.54	5.00
D	150	0.10	50	250	0.00	70	285	2.40	5.00

2.8. MS detection and data handling

The transfer line, manifold, and ion source operated at 275, 40, and 200 °C, respectively. Ionization and fragmentation were achieved by electronic impact (EI) at 70 eV. A full scan MS experiment was performed by the first quadrupole (Q1), as preliminary tests showed it had a higher sensitivity than the third quadrupole (Q3) (data not shown). The mass range of the full scan acquisition was 40–550 amu.

The chromatograms acquired in the GC–MS mode were processed using deconvolution software available online, AMDIS (NIST, Version 2.68). The deconvolution and identification parameters were as follows: minimum match factors: 50; type of analysis: simple; component width: 12; adjacent peak subtraction: two; resolution: high; sensibility: very high; shape requirement: low. For identification, the MPW2011 library [32] was merged into an AMDIS library along with the pure spectra of any missing or poorly identified compounds acquired with our instruments.

Selected reaction monitoring (SRM) transitions for the MS–MS experiments were developed for all 54 compounds. For details, see Table 2.

2.9. Evaluation of the method

The occurrence of false positives was investigated by injecting 10 different urine samples as negative controls according to immuno-assays and routine GC–MS screening.

The limits of detection (LODs) were evaluated by injecting the extracts from urine samples spiked with decreasing drug concentrations (500, 250, 100, 50, 25, 10, 5, 1 ng/mL). The same extracts were subsequently injected into the GC–MS and corresponding GC–MS/MS systems. The GC–MS LODs were taken as the lowest concentration that yielded an occurrence of the analyte on the AMDIS result table. The GC–MS/MS LODs were evaluated as the minimum concentration to yield a signal-to-noise ratio greater than 3 for both of the SRM transitions.

The stability of the analytical system was tested by monitoring the peak area and retention time of the IS for 40 serially injected samples.

To evaluate the overall screening method, 139 urine samples were analyzed, and the results were compared to those of the routine investigations. The routine STA procedure consists of two steps: i. immunoassays (EMIT®) for amphetamines, cannabis, cocaine, LSD, opiates, barbiturates, benzodiazepines, methadone, buprenorphine, propoxyphene and tricyclic antidepressants; ii. GC–MS screening of 500–1000 μL of urine extracted at a pH of 9 with n-chlorobutane and then acetylated. These urine samples were only hydrolyzed with glusulase (heating at 60 °C during 3 h) if either opiates or benzodiazepines were detected by the immunoassays prior to extraction. The acetylated extracts were injected into an Agilent 6890Series gas chromatograph coupled to an Agilent 5973Network mass spectrometer. The chromatographic run lasted 30 min. The acquired spectra were manually compared to the same databases as used for the presented method with spectral purification accomplished through manual background subtraction.

The detection performance of GC–MS/MS was evaluated for cannabinoids and buprenorphine, as these compounds tend to be difficult to detect by GC–MS. Immunoassay calibrators corresponding to the cut-off values, i.e., 50 ng/mL for cannabinoids and 5 ng/mL for buprenorphine, were injected into the GC–MS/MS using the corresponding method along with authentic urine samples that had given positive and negative results for cannabinoids ($n=10$) and buprenorphine ($n=3$). Samples were considered positive if both SRM transitions yielded peaks at the correct retention time, with a signal-to-noise ratio greater than 3.

Table 2
SRM transitions, collision energy (CE), limit of detection (LOD) for both GC–MS and GC–MS/MS screening methods and the retention time (RT) corresponding to the respective GC–MS/MS program for 54 drugs of interest. Acetylated and diacetylated compounds are indicated by the labels (Ac.) and (2Ac.), respectively.

	Compound	SRM transition (CE)	LOD GC–MS	LOD GC–MSMS	RT GC–MSMS
			(ng/mL)	(ng/mL)	(min)
Group A	Cocaine	303→82 (−25 V) 182→82 (−10 V)	50	2	3.01
	Cocaethylene	317→82 (−15 V) 196→82 (−15 V)	25	2	3.10
	Methylecgonine (Ac.)	241→82 (−10 V) 182→82 (−10 V)	25	1	2.11
	Morphine (2Ac.)	369→327 (−10 V) 327→268 (−10 V)	50	1	4.05
	Codeine (Ac.)	341→282 (−10 V) 282→266 (−10 V)	25	1	3.74
	Methadone	294→223 (−10 V) 72→57 (−20 V)	50	2	3.02
	EDDP	277→105 (−15 V) 262→105 (−10 V)	50	2	2.72
	Fentanyl	245→146(−15 V) 146→131 (10 V)	50	5	4.13
	Oxycodone (Ac.)	357→298 (−10 V) 314→201 (−15 V)	100	5	3.63
	Oxymorphone (Ac.)	343→284 (−10 V) 300→141 (−30 V)	100	5	4.11
	Dihydrocodeine (Ac.)	343→284 (−10 V) 226→211 (−10 V)	50	2	3.51
	Tramadol (Ac.)	305→58 (−15 V) 188→134 (−10 V)	25	<1	2.70
	Meperidine/Pethidine	247→71 (−10 V) 172→91 (−15 V)	50	2	2.27
	Hydrocodone	299→242 (−10 V) 242→185 (−20 V)	25	2	3.50
	THC–COOH (Ac.)	400→343 (−15 V) 343→283 (−15 V)	>500	5	4.41
	Ketamine (Ac.)	216→56 (−15 V) 208→138 (−15 V)	25	1	3.03
	Diphenhydramine	165→115 (−30 V) 152→126 (−15 V)	100	5	2.47
Group B	Buprenorphine (Ac.)	509→452 (−5 V) 420→281 (−15 V)	500	1	4.28
	Norbuprenorphine (2Ac.)	440→408 (−5 V) 408→366 (−5 V)	>500	1	4.65
Group C	Amphetamine (Ac.)	177→118 (−25 10 V) 118→91 (−25 V)	25	<1	3.34
	Methamphetamine (Ac.)	191→100(−10 V) 100→58 (−10 V)	50	1	3.47
	MDMA (Ac.)	235→162 (−5 V) 162→104 (−15 V)	25	<1	4.14
	MDE (Ac.)	249→162 (−15 V) 114→72 (−10 V)	25	2	4.19
	MDA (Ac.)	221→162 (−10 V) 162→104 (−15 V)	50	<1	4.00
	mCPP (Ac.)	238→154 (−10 V) 154→118 (−15 V)	25	<1	4.57
Group D	Alprazolam	308→273 (−5 V) 279→243 (−15 V)	100	<1	4.61
	Bromazepam (Ac.)	315→208 (−20 V)	200	25	3.47
	Clonazepam	315→288 (−5 V) 280→234 (−20 V)	50	2	4.19
	Diazepam	284→256 (−10 V) 256→221 (−10 V)	25	<1	2.94
	Flunitrazepam	313→286 (−5 V) 286→240 (−10 V)	50	<1	3.34
	Flurazepam	387→86 (−10 V) 86→58 (−10 V)	50	<1	3.81
	Hydroxymidazolam (Ac.)	383→340 (−10 V) 310→95 (−30 V)	50	<1	4.14
	Lorazepam (Ac.)	404→389 (−10 V) 347→291 (−10 V)	50	2	3.91
	Lormetazepam (Ac.)	376→334 (−5 V) 305→193 (−15 V)	25	1	3.83
	Midazolam	325→310 (−10 V) 310→95 (−30 V)	25	<1	3.2
	Nordazepam (Ac.)	270→242 (−10 V) 242→207 (−10 V)	25	<1	3.11
	Oxazepam	286→257 (−10 V)	50	<1	3.25

Table 2 (*continued*)

		257→77 (−20 V)			
	Temazepam (Ac.)	342→257 (−10 V)	25	<1	3.54
		271→193 (−15 V)			
	Zolpidem	307→235 (−10 V)	25	<1	3.91
		235→92 (−20 V)			
	Zopiclone	245→217 (−10 V)	100	<1	4.70
		143→99 (−10 V)			
Group E	Amitriptiline	215→189 (−10 V)	50	<1	3.01
		202→152 (−25 V)			
	Citalopram	324→58 (−15 V)	50	<1	3.39
		238→218 (−10 V)			
	Clozapine	326→243 (−10 V)	50	<1	4.88
		243→208 (−15 V)			
	Imipramine	280→235 (−10 V)	50	<1	3.06
		234→218 (−25 V)			
	Mirtazapine	265→195 (−10 V)	20	<1	3.12
		195→167 (−30 V)			
	Fluoxetine (Ac.)	190→86 (−10 V)	25	<1	3.11
		117→91 (−15 V)			
	Fluvoxamine (Ac.)	258→226 (−10 V)	100	5	3.06
		102→60 (−5 V)			
	Reboxetine (Ac.)	218→176 (−10 V)	25	<1	3.99
		176→91 (−15 V)			
	Sertraline (Ac.)	347→274 (10 V)	50	<1	4.44
		290→144 (−10 V)			
	Trimipramine	294→249 (−10 V)	25	<1	3.03
		249→234 (−10 V)			
	Venlafaxine	202→121 (−10 V)	25	<1	2.90
		134→91 (−20 V)			
	Carbamazepine (Ac.)	236→193 (−10 V)	25	1	3.30
		193→165 (−10 V)			
	Haloperidole (Ac.)	237→98 (−10 V)	100	<1	4.43
		224→84 (−10 V)			
	Olanzapine (Ac.)	354→272 (−10 V)	100	5	4.32
		284→242 (−10 V)			

3. Results and discussion

3.1. Microwave-assisted enzymatic hydrolysis

The hydrolysis of glucuronide conjugates is unavoidable for compounds such as morphine, codeine, buprenorphine and some benzodiazepines because the concentrations of these analytes in their free form in urine is often too low to be detectable by GC–MS [20].

Our objective was to develop a 5 min enzymatic hydrolysis step prior to GC–MS screening with conventional instrumentation. This means that the complete hydrolysis of glucuroconjugates is not expected as only a sufficient cleavage of the conjugated derivatives to form the free analytes to enable their detection is required.

Domestic instruments do not allow for the fine tuning of the irradiation conditions [24]. In fact, only the average irradiation power (90, 160, 350, 500, 650, 750 and 850 W) can be selected. Preliminary tests showed that an irradiation of over 500 W could not be achieved in the described configuration, as the plastic micro-tubes were not sufficiently resistant. A 5 min irradiation at 350 W was found to produce the best hydrolysis yields.

The hydrolysis efficiency was evaluated using authentic urine samples. Unhydrolyzed extracts were injected to evaluate the free-form analyte concentration, while samples hydrolyzed for 3 h at 60 °C were analyzed to calculate the maximum hydrolysis yield. This strategy does not allow for the absolute evaluation of the microwave-assisted enzymatic hydrolysis as the exact concentrations of the conjugated analytes was not known; however, it is sufficient for our objective of qualitative analysis.

Fig. 1 shows the hydrolysis yields calculated for morphine, codeine, buprenorphine, oxazepam and temazepam from 8 real urine samples. Satisfactory performances were observed for buprenorphine and the two selected benzodiazepines, which had hydrolysis yields from 60% to 94%. Hydrolysis of opiates proved to be less efficient, with yields from 10% to 46%. These compounds could, however, still be detected by AMDIS from the GC–MS data after the rapid hydrolysis protocol. In fact, the estimated analyte concentrations before and after hydrolysis showed that the hydrolysis increased the free-form concentration to values above the GC–MS LODs. Great variability was observed for the hydrolysis yields of the different urines, which could not be reduced by adjusting the pH to 5.2 using acetic acid as described elsewhere [18]. We concluded that other parameters,

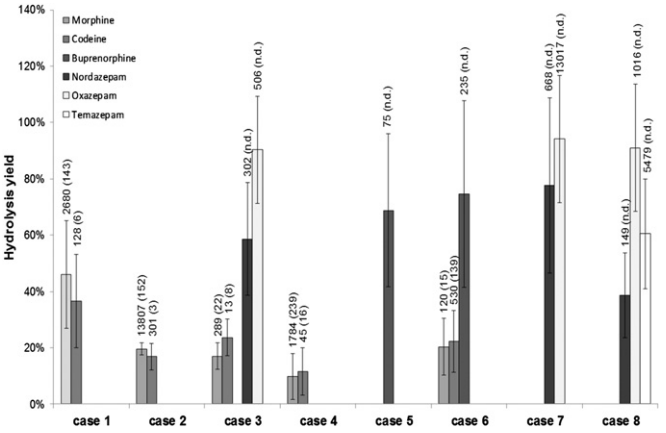

Fig. 1. Hydrolysis yields expressed as the microwaves hydrolysis vs. oven hydrolysis ratio and calculated for the compounds present in 8 real urine samples treated in triplicates ($n=3$). The estimated total concentrations are indicated over the corresponding bars, and the estimated free-form concentrations are indicated in brackets. Values are expressed in ng/mL.

such as the protein concentration, might influence the enzymatic reaction in urine. However, the hydrolysis procedure was found to be satisfactory for qualitative purposes based on the time it saved.

3.2. LLE

LLE was chosen as the simplest and most general screening extraction method [20]. Unfortunately, the nature of the chosen solvent and working pH for the extraction greatly influenced the drug recoveries, and the development of a general extraction procedure remains a vain wish. Therefore, conditions corresponding to the most commonly encountered drugs in forensic toxicology, i.e., low polarity basic compounds, were chosen and applied to all of the drug classes. To reduce the amount of solvent and evaporation time required, the volume of the extracted urine was reduced to 200 μL, which allowed the use of only 1 mL of the extractive solvent or solvent mixture.

The performance of the extraction mixture was not evaluated based solely on the extraction recoveries, but also the cleanness of the extracts. This is an important factor for drug detection and system robustness. The recoveries of the four tested solvent mixtures are showed in Table 3. According to these values, the best extraction solvent mixture was EA:IsoPrOH:CH_2Cl_2 3:1:1 v/v/v at pH=9. However, this extraction mixture produced unclean extracts. BuCl allows for the good extraction of low polarity compounds and provides cleaner residues than EA:IsoPrOH:CH_2Cl_2. However, the recoveries of some analytes of interest, such as morphine, methylecgonine or methadone, were not satisfactory, which could lead to false negatives. Adding small portions of isopropanol enhanced the extractive power of n-chlorobutane without reducing the extract cleanliness. The recoveries for BuCl:IsoPrOH were less than for EA:IsoPrOH:CH_2Cl_2, with the exception of methadone, but these recoveries were still acceptable.

CH_2Cl_2:Hexane:EA yielded very unclean extracts with lower recoveries than EA:IsoPrOH:CH_2Cl_2, with the exception of THC–COOH, which requires a strong apolar solvent such as hexane for extraction.

Considering these results, BuCl:IsoPrOH 4:1 v/v was found to be the best compromise between good recoveries for the analytes of interest and a clean extract.

3.3. GC–MS screening

Reducing the sample preparation time makes no sense if the analysis time is not shortened as well. The use of deconvolution software improves the identification of coeluted compounds. Thus, as a high chromatographic resolution is no longer critical, a faster GC run can be implemented. A conventional GC–MS apparatus was used with a 0.25 μm ID capillary column, which improved the analytical capacity and enabled splitless injections. The analysis time was then reduced a rapid temperature program (upto 70 °C/min gradient, instrument maximum capability), as currently performed in our laboratory for quantitative purposes [33,34].

The initial temperature was set to 85 °C to avoid the rapid elution of any volatile analytes of interest, such as acetylated amphetamines. Despite the initial oven temperature being above the reconstitution solvent boiling point, the peak shapes of first compounds eluted were found to be satisfactory (Table 4). The final temperature was set to 285 °C and held 5.5 min. Therefore, over half of the GC run was devoted to eluting low-volatility compounds. This step was necessary to restore the column and avoid inter-run carryover because the nature of the injected extracts and full scan data acquisition makes STA methods very sensitive to contamination.

Monitoring trimipramine-d3 in over 40 injections of urine extracts indicated that the system was stable. Indeed, no significant decrease in the area of the *m/z* 249 peak was observed. Moreover, the retention time of trimipramine-d3 was very reproducible, with a standard deviation below 1%. Finally, no false positives were detected upon the injection of pure methanol at

Table 4
Retention times, peak widths and AMDIS net identification match factors for 12 selected compounds spiked in urine at 500 ng/mL and both extracted and injected using the 10 min full scan GC–MS program. Acetylated and diacetylated compounds are indicated by the labels (Ac.) and (2Ac.), respectively.

Compounds	Retention time (min)	Peak width (min)	AMDIS net identification match factor
Amphetamine (Ac.)	3.25	0.08	92
Methylecgonine (Ac.)	3.49	0.06	80
Phenobarbital	4.24	0.06	75
Venlafaxine	4.49	0.06	76
Methadone	4.50	0.06	90
Codeine (Ac.)	5.22	0.06	90
Nordiazepam	5.26	0.06	93
Morphine (2Ac.)	5.52	0.06	94
Bromazepam (Ac.)	5.60	0.09	55
Zolpidem	6.10	0.08	93
Hydroxymidazolam (Ac.)	6.29	0.07	89
Clozapine	6.45	0.07	95

Table 3
Overall extraction recoveries for selected model compounds spiked into blank urine at 500 ng/mL. The pH was set to 9 using a borate buffer before extracting 200 μL with 1 mL of the corresponding solvent mixture.

Compounds	Extraction mixture							
	CH_2Cl_2:Hexane:EA 5:4:1 v/v		EA:IsoPrOH:CH_2Cl_2 3:1:1 v/v		BuCl		BuCl:IsoPrOH 4:1 v/v	
	Recovery (%)	%RSD	Recovery (%)	%RSD	Recovery (%)	%RSD	Recovery (%)	%RSD
Amphetamine	63	9	104	7	35	10	77	9
Clozapine	88	8	102	5	98	5	103	9
Codeine	65	3	99	5	59	5	88	8
Methadone	41	8	78	2	40	8	92	1
Methylecgonine	43	8	73	1	16	5	48	6
Morphine	22	4	89	6	1	10	50	5
Nordiazepam	90	1	103	10	104	2	104	7
Hydroxymidazolam	77	2	104	9	76	4	73	7
Phenobarbital	42	8	103	9	7	9	13	8
THC–COOH	40	3	17	6	8	9	11	14
Venlafaxine	90	9	93	2	73	5	88	6
Zolpidem	83	4	98	8	69	4	65	5

the end of the analysis batch, even after a large number of sample injections.

To ensure that the proposed temperature program is suitable and that the chromatographic separation was sufficient for identification, the compound mixture used for extraction optimization was analyzed. The AMDIS deconvolution and identification parameters were set as described by Meyers et al. [10], with the exception of the component width, which was set to 12 scans. The retention times, peak widths and AMDIS net identification match factor for each model compound are presented in Table 4. The resolution appeared to be sufficient for all compounds except methadone and acetylated venlafaxine. These two analytes overlapped, with a retention time difference of 0.01 min. However, AMDIS was able to distinguish them and provided the correct identifications with satisfactory match factors.

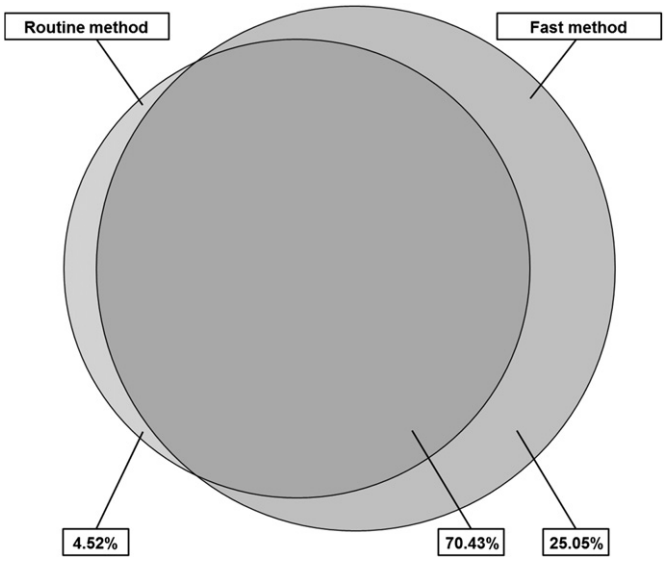

Fig. 2. Euler diagram representing the distribution of the 487 positive matches obtained from 139 urine samples analyzed using both the routine and presented rapid methods.

The 10 min AMDIS-assisted screening method was evaluated by extracting and analyzing urine samples from 139 patients, which included 19 postmortem samples, and comparing the identifications to the screening results obtained using the routine procedure. The inter-method similarities and differences observed for each sample are summarized in a Euler diagram (Fig. 2). Nicotine and its metabolites; paracetamol and its metabolites; and caffeine, theobromine and theophylline were not accounted for to reduce the number of compound entries. These frequently occurring analytes may be important for analytical and toxicological interpretations; however, their detection and identification are generally unproblematic. A total of 487 identifications were made for the 139 analyzed samples, which included 343 corresponding matches in both methods: 22 exclusively found by the routine method and 122 exclusively found by the fast method. The presented method proved to be considerably more sensitive to opiates than the routine method, as was expected based on the extraction investigations. However, in two cases both morphine and codeine were only detected by the routine method. Fentanyl was also missed twice by the fast screening. These compounds were, however, detected in

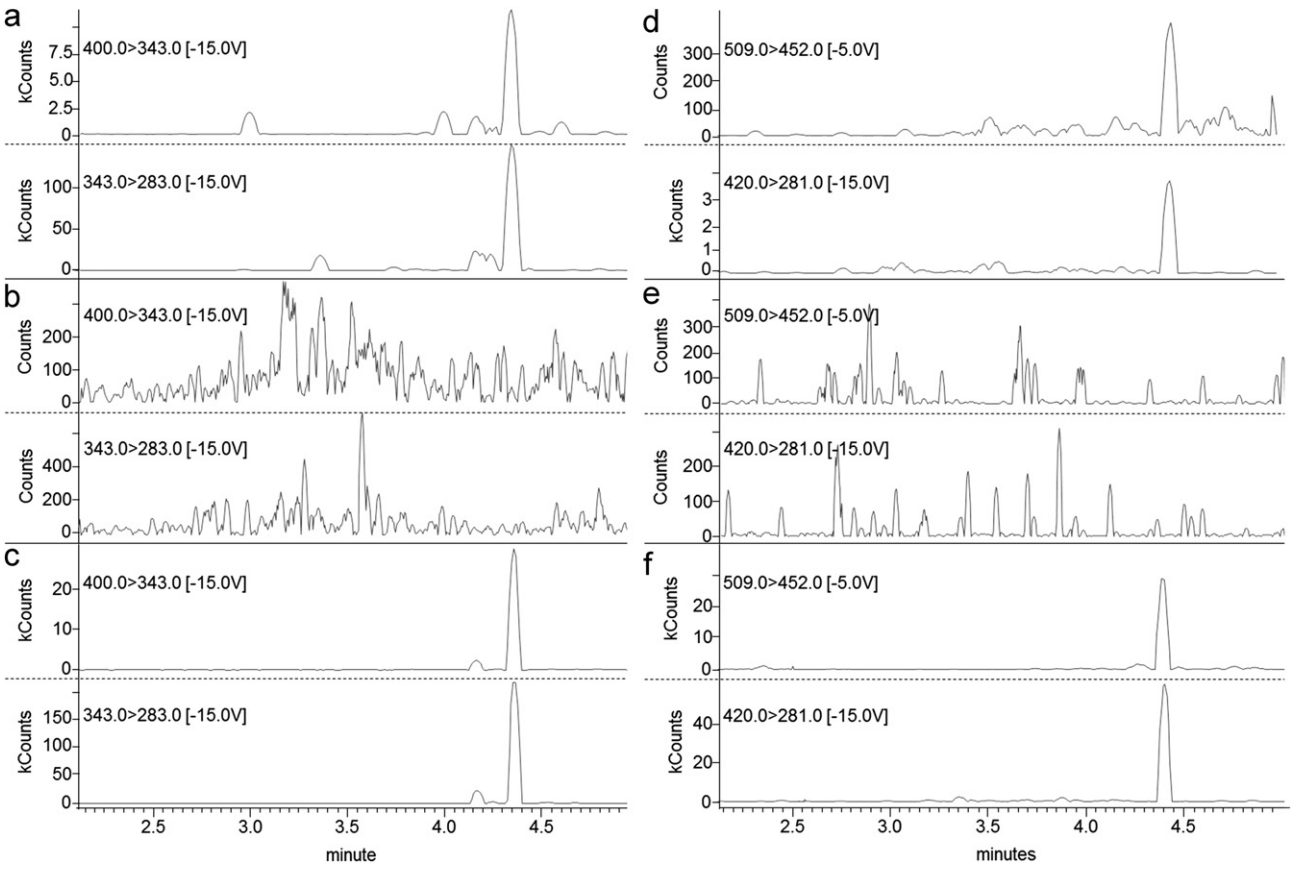

Fig. 3. Ion current chromatograms for (a) a 50 ng/mL cannabinoids immunoassay calibrator (cut-off level), (b) an extracted cannabinoids-negative (EMIT®) urine sample, (c) an extracted cannabinoids-positive urine sample, (d) a 5 ng/mL buprenorphine immunoassay calibrator (cut-off level), (e) a buprenorphine-negative urine sample and (f) a buprenorphine-positive urine sample.

the GC–MS/MS screening. Benzodiazepines were also more often identified by the fast screening thanks to the improved extraction of the mass spectral data by the deconvolution software. Over the 22 compounds identifications provided exclusively by the routine method, 9 were caused by variations of benzodiazepines identifications. This is probably due to the different algorithms used for the spectral comparison. In one postmortem case, gabapentin was identified by the fast method, whereas valproic acid was identified by the routine method. Unfortunately, the low sample amounts did not allow for confirmatory testing.

Taking these differences into account, the fast method presented here proved to have detection and identification capabilities comparable to the routine method.

3.4. GC–MS/MS target screening

Using a triple quadrupole instrument enables an important sensitivity increase. This increased sensitivity is of interest for problematic compounds such as cannabinoids and buprenorphine, which are generally present at very low concentration or do not respond well to GC–MS screenings, and cannot be identified with satisfactory match factors. Therefore, MS/MS may be an interesting tool for STA, which may improve the reliable identification of the drugs contained in a sample.

The SRM transitions were developed to be compatible with the extract injected during the GC–MS screening. This means that only acetylation was considered for compounds with polar moieties, even if other types of derivatization agents would have improved the sensitivity. This strategy avoided having to prepare two different extracts for the GC–MS and GC–MS/MS screenings, which saves time.

Different acquisition methods were developed for several class of drug, as the system was unable to monitor all of the transitions in a single run. This allowed for the optimization of the temperature programs for the 5 constituted compound families (Table 2). It was then possible to limit the analysis time to 5 min.

As shown in Table 2, the use of MS/MS acquisition substantially increased the sensitivity for the selected compounds, with LODs upto 100 times lower than those for simple MS.

Fig. 3 shows chromatograms corresponding to the calibrators used for the immunoassays at their cut-off levels and urine samples that were determined to be positive for cannabinoids and buprenorphine by immunoassays. GC–MS/MS target screening discriminated between the positive and negative samples using the selected cut-off, as peaks with signal-to-noise ratios over 3 appeared for both transitions at their corresponding retention time. All of the samples analyzed by GC–MS/MS gave results in concordance with the immunoassay screening. This means that this strategy avoids the need for expensive time and sample consuming immunoassay testing for these compound classes.

4. Conclusion

In conclusion, our results showed that the sample preparation time before urine screening can be significantly reduced by using microwave energy and low urine volumes. Moreover, the use of AMDIS for data analysis allowed the analytical run to be accelerated without impairing the quality of the results. Finally, tandem mass spectrometry appeared to be a cheaper and quicker alternative to immunoassays for the rapid and sensitive detection of drugs in toxicological screening applications. The development of several analysis methods adapted to a single urine sample extraction proved to be very convincing, as it improved the analytical performance in terms of drug detection while reducing the amount of sample and sample preparation time required.

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