Journal of Analytical Toxicology, 2023, **47**, 33–42
DOI: https://doi.org/10.1093/jat/bkac020
Advance Access Publication Date: 26 March 2022
Article

Development of a Liquid Chromatography–Tandem Mass Spectrometry Method for the Simultaneous Determination of 40 Drugs of Abuse in Human Urine: Application to Real Cases

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Abstract

Drugs of abuse are constantly evolving, while new synthetized substances are constantly emerging to avoid regulations. However, traditional drugs such as cocaine and amphetamine are still two of the most consumed drugs in the world. It is important, therefore, to provide suitable multiresidue methods for determining a wide range of drugs for use in toxicological and forensic analyses. The aim of this study is to develop a method for determining several families of drugs of abuse, including classic drugs, new psychoactive substances and some of their metabolites, in urine by liquid chromatography–tandem mass spectrometry. Urine is one of the most common biological matrices used in drug analysis because of its easy collection and a wide window of detection. In this study, we used solid-phase extraction to remove interferences and extract analytes from urine. Four different mixed-mode cation-exchange commercial sorbents were evaluated. The best results, in terms of apparent recoveries, were achieved with one of the strong cationic sorbents, ExtraBond SCX. The method achieved detection limits from 0.003 to 0.500 ng/mL and quantification limits from 0.050 to 1.500 ng/mL, which are suitable for determining these compounds at the usual levels found in the urine of drug users. The applicability of this method was demonstrated by analyzing real urine specimens from women following a detoxification program. Our results showed that the drug most consumed was cocaine, since it was detected in most urine specimens together with its main metabolite, benzoylecgonine. The polyconsumption of drugs from different families was also observed in some urine samples analyzed.

Introduction

The last few years have seen the wide availability of numerous substances on the drug market. Apart from classic drugs, there has been a high demand for more potent substances, which is where synthetic drugs play an important role. Moreover, the prohibition of certain substances has led to the consolidation of illegal markets. Hundreds of new psychoactive substances (NPSs), especially stimulant substances, have been synthetized (1). In view of these new substances, as well as the classic drugs already on the global market, drugs of abuse (DOAs) are now widely available. These can be classified in different ways. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported the following classification in its annual report: cannabis, cocaine (COC), amphetamine-type substances (ATSs), NPS, heroin (HER) and other opioids, and other drugs such as ketamine (KET) and lysergic acid diethylamide (LSD) (2). According to the EMCDDA, the amounts of COC seized in Europe are now the highest ever seen, and this drug has become the second most consumed illicit drug in Europe after cannabis. Another well-known group is ATS, which contains substances such as amphetamine (AMP), methamphetamine (MAMP) and 3,4-methylenedioxymethamphetamine (MDMA or ecstasy) (3). In addition to ATS, COC and cannabis, other substances also consumed in Europe are LSD, KET and NPS. The latter is a heterogeneous group of synthetic drugs in which the most detected substances are synthetic cannabinoids, synthetic cathinones and synthetic opioids (4, 5). Control of DOAs is a matter of concern, and Spain is an important country for its interception because it is one of the main gateways for the entry of COC and cannabis into Europe (3–5). Several studies in Spanish wastewaters, for example, have revealed an increase in ATS and COC consumption and high levels of COC metabolites in wastewater samples (6–8).

These substances are controlled by medical and government authorities, who are interested in developing reliable multiresidue methods that can simultaneously determine a large number of DOAs, including classic drugs and new synthetic substances. In forensic and toxicological analyses, their determination is important in biological matrices such as urine, blood, oral fluid or hair (4, 9, 10). Urine has several advantages over other biological matrices because it provides a wide window of detection (from minutes to days or weeks), in general, no specialized personnel is required for the sampling and the amount of sample produced is very high. It is also important sensitive methods able to determine both

the parent drugs and their metabolites, which can be found between low and high levels of ng/mL. Urine also has several drawbacks, however, including easy adulteration when the collection is not observable and invasive sampling when it is observable (9–15).

The complexity of urine makes it important to conduct sample pretreatment before analysis because possible interferents with the analytes of interest can be problematic (9, 10, 15). One of the most common sample pretreatments in the literature is solid-phase extraction (SPE). This has been used by numerous authors with methods for determining various types of DOAs with different SPE sorbents (16–20). Since most drugs are basic compounds with pKa values >7, at a lower pH they are positively charged and mixed-mode cationic-exchange sorbents can therefore be perfect candidates (9, 19). A wide range of commercial cation-exchange SPE sorbents exists. It is important to choose the one that provides the highest recovery efficiencies for most of the compounds studied. In this paper, we compare four different commercial mixed-mode cation-exchange sorbents for sample pretreatment.

To analyze the extracted urine analytes, various techniques can be used, including liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (9). The most common of these is LC because of the polar characteristics of the DOAs under study. In general, LC has been combined with mass spectrometry (MS) and, more precisely, with tandem MS (MS-MS) using a triple quadrupole (QqQ) or QTrap or with LC coupled to high-resolution mass spectrometry (HRMS) by using Orbitrap to obtain sensitive and selective methods (14, 16–19, 21–28). Concheiro et al. (25), for example, developed a method for determining 40 novel psychoactive stimulants in urine by LC–QOrbitrap using SOLA SCX cartridges for the SPE urine pretreatment and achieved extraction efficiencies between 90% and 100% for most compounds. The authors obtained limits of detection (LODs) between 1.0 and 5.0 ng/mL and limits of quantification (LOQs) between 2.5 and 5.0 ng/mL. For their part, Rosano et al. (14), Ambach et al. (24) and Di Trana et al. (26) used LC–MS-MS to determine DOAs in urine using sample pretreatment procedures such as hydrolysis, liquid–liquid extraction (LLE) and dilute-and-shoot, respectively. In particular, when Di Trana et al. (26) analyzed urine specimens from drug users, they determined morphine (MOR) (180 ng/mL), several synthetic ATSs (e.g., 6-APB from 3.5 to >10,000 ng/mL) and several synthetic cathinones (e.g., mephedrone between 12.4 and 3,597 ng/mL), among other substances. These results and those of similar studies show the wide concentration ranges at which these substances can be found in urine and the need for sensitive methods to determine them. Despite the good results generally obtained with the above methods, most strategies focus mainly on a low number of drugs generally belonging to the same family. It is important, therefore, to provide methods that can simultaneously determine different classes of drugs, including classic drugs and the new synthetic drugs, because polyconsumption is very common among drug users. The main aim of this study is to contribute to the literature with a fast and sensitive method for the multiclass determination of different types of DOAs and some of their metabolites in urine by SPE followed by LC–QqQ.

This study focuses on some of the most consumed classic illicit drugs and some NPS (e.g., synthetic cathinones). To extract these drugs from urine and perform clean-up, we tested and compared for the first time several types of mixed-mode cation-exchange sorbents (both weak and strong), including Oasis WCX, Oasis MCX, Extrabond ECX and ExtraBond SCX. Finally, to prove that this is a useful method in toxicological cases, we applied it to urine samples from women following a detoxification program.

Experimental

Standards and materials

The following drug standards were purchased from LGC Standards (Luckenwalde, Germany) or Sigma Aldrich (St. Louis, MO, USA): 4-fluoromethcathinone (flephedrone), *N*-ethylcathinone (ethcathinone), buphedrone,2-methylmethcathinone (2-MMC), butylone, mephedrone, 4-methylethcathinone (4-MEC), beta-ethylmethcathinone (pentedrone), 3,4-dimethylmethcathinone (3,4-DMMC), alpha-pyrrolidinovalerophenone (alpha-PVP), methylenedioxypyrovalerone (MDPV), 3,4-methylenedioxymethcathinone (methylone), 4-methoxymethcathinone (methedrone), 3,4-methylenedioxy-*N*-ethylcathinone (ethylone), dimethylcathinone (DMC), pyrovalerone, AMP, MAMP, MDMA, 3,4-methylenedioxyamphetamine (MDA), COC, benzoylecgonine (BZE), MOR, 6-acetylmorphine (6-AM), buprenorphine, HER, fentanyl, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), codeine (COD), alprazolam, lorazepam, diazepam, bromazepam, KET, norketamine, LSD, *oxo*-LSD, 4-methylephedrine and hyoscine.

Depending on the compound, individual stock solutions at 100, 1,000 and 2,000 mg/L were prepared in methanol (MeOH) and frozen at −20°C. A mixture solution at 2 mg/L in MeOH was also prepared with all the individual standards and kept in the freezer. From this mixture, diluted working solutions in water (H_2O) were further prepared.

H_2O and acetonitrile (ACN) for LC–MS were obtained from Scharlab (Barcelona, Spain). MeOH was purchased from J.T. Baker (Deventer, The Netherlands). Formic acid (HCOOH) ≥98%, hydrochloric acid (HCl) ≥37%, ammonium hydroxide (NH_4OH), sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained from a water purification system (Merck Millipore, Darmstadt, Germany).

Several extraction cartridges for SPE were evaluated. Oasis WCX and MCX (150 mg/6 mL) were purchased from Waters Corp. (Milford, MA, USA). ExtraBond ECX (200 mg/6 mL) and ExtraBond SCX (1,000 mg/6 mL) were purchased from Scharlab. A MiVAc Duo sample concentrator from Genevac (Ipswich, UK) was used to evaporate the solutions.

LC–MS-MS conditions

For the analysis, we used an Agilent model 1200 series LC coupled with an Agilent 6460 series triple quadrupole mass spectrometer with an electrospray ionization interface from Agilent Technologies (Waldbronn, Germany). Agilent MassHunter Workstation Software version B.09.00 was used for instrumental control and data analysis.

The column used for chromatographic separation was a Luna Omega 5-µm Polar C_{18} (150 mm × 4.6 mm, 5 µm) from Phenomenex (Torrance, CA, USA) with a Security Guard from Phenomenex. A mobile phase flow rate of 0.6 mL/min was used with an injection volume of 10 µL. The mobile phase was A: 0.1% HCOOH in H_2O and B: 0.1% HCOOH in ACN in gradient mode. The gradient was as follows: initial 15% B was maintained for 5 min before increasing to 35% in 7 min and to 80% in 13 min. It was then increased to 100% in 1 min and held for 2 min before returning to the initial conditions in 1 min, where it was maintained for 5 min.

The MS-MS parameters were optimized by injecting each compound at 1 mg/L in a mixture of H_2O:MeOH (50:50, v/v). The acquisition was performed in multiple reaction monitoring (MRM) mode in positive polarity. The drugs were divided into 10 different windows depending on their retention times (R_t). The optimal MS-MS parameters were as follows: gas temperature, 350°C; gas flow rate, 13 L/min; nebulizer pressure, 30 psi and capillary voltage, 2,500 V. The fragmentor was set between 50 and 125 V, and the collision energy (CE) was set between 4 and 42 eV. The two most intensive transitions between the parent ion and the product ions were selected for the MRM mode (Table I).

Urine collection and preparation

Urine samples were collected in polypropylene tubes and kept in the freezer at –20°C. Before analysis, the samples were thawed, and 5 mL of a mixture of urine and ultrapure water adjusted to pH 4 with HCOOH ≥98% at a ratio of 50:50 (v/v) was loaded into a 1.5-g ExtraBond SCX cartridge after activation with 5 mL of MeOH and conditioning with 5 mL of ultrapure water at pH 4. This was then washed with 2 mL of MeOH, and the analytes were eluted with 3 mL of 5% NH_4OH in MeOH. After the SPE procedure, 100 µL of 1% HCl ≥37% in MeOH was added to the methanolic solution, and the extracts were evaporated to dryness with a MiVac sample concentrator. Finally, the extracts were reconstituted with 0.5 mL of mobile phase at initial conditions, filtered through a 0.45-µm polytetrafluoroethylene (PTFE) syringe filter and transferred to a vial for analysis.

Validation

The method was validated by following the European guidelines for workplace drug testing in urine (29). The validated parameters were linearity, sensitivity, instrumental detection and quantification limits (IDLs and IQLs), method detection and quantification limits (MDLs and MQLs), apparent recoveries (R_{app}), matrix effect (ME), precision as repeatability (intra-day) and reproducibility (inter-day), selectivity, stability and accuracy. Tolerances of ±2.5% and ±20% were also considered for retention times and ion ratios, respectively (30).

Instrumental linearity was evaluated by using different working solutions of drug neat standards between 0.010 and 250 ng/mL and considering the determination coefficient (r^2). IDLs were defined as the lowest detectable point with a signal-to-noise (S/N) ratio ≥3. IQLs were defined as the lowest concentration in the calibration curve with an S/N ratio ≥10.

Method linearity was studied by using matrix-matched calibration curves with a mixture of drugs in urine at concentrations between 0.010 and 125 ng/mL. The criteria for method linearity, MDLs and MQLs were the same for the extracted samples as they were for the instrumental values. R_{app}, ME, repeatability and reproducibility were studied at 2 (low), 20 (medium) and 65 ng/mL (high) concentration levels using five replicates ($n = 5$). Repeatability ($n = 5$ on the same day) and reproducibility ($n = 5$ for 5 days) were studied in terms of relative standard deviation (%RSD).

Selectivity and specificity were studied by evaluating possible endogenous and exogenous interferences from urine. Stability was studied by analyzing replicates of the three calibrator levels during the sequence (2, 20 and 65 ng/mL) in urine for 50 h at room temperature ($n = 5$). Accuracy was studied in terms of error by analyzing three blind samples spiked at various concentrations before conducting the experimental procedure and comparing the concentrations obtained to the real ones.

Analysis of real samples

Urine samples from 22 anonymous women who began a drug-abuse detoxification process were provided by the Centre Català de la Solidaritat (CECAS) in Tarragona, Spain. All samples were collected in polypropylene tubes when the patient was admitted. The samples were then frozen at –20°C before the sample pretreatment step described earlier was conducted.

Results and Discussion

Separation and detection

Forty compounds, including DOAs and some of their metabolites, were separated with a Luna Omega 5-µm Polar C_{18} column (150 mm × 4.6 mm i.d., particle size 5 µm). The metabolites included in this study were 4-methylephedrine (metabolite of mephedrone), AMP (MAMP), MDA (MDMA), BZE (COC), norketamine (KET), EDDP (methadone), 2-*oxo*-LSD (LSD) and 6-AM (HER). The mobile phase comprised A: 0.1% HCOOH in H_2O and B: 0.1% HCOOH in ACN. This selection was based on studies previously conducted by our research group in which several synthetic cathinones were determined in various biological matrices (19, 31). The elution gradient was also based on the strategies employed in those studies. However, as the present study included more DOAs, it was modified to obtain the most adequate separation of all the compounds. The mobile phase therefore began at 15% B, which was maintained for 5 min before increasing to 35% in 7 min. This was increased to 80% in 13 min and then to 100% in 1 min, where it was held for 2 min before returning to the initial conditions in 1 min and being held for 5 min. The flow rate for the mobile phase was 0.6 mL/min with an injection volume of 10 µL. Under these chromatographic conditions, most DOAs were separated in <25 min.

To evaluate the MS-MS parameters, we used standard solutions of each drug at 1 mg/L. The fragmentor voltage was tested between 50 and 200 V, and the CEs were tested between 0 and 50 kV. The two most abundant transitions of each drug were chosen to confirm their identification in positive mode (Table I). The source conditions, capillary voltage (2,500 V), gas temperature (350°C), gas flow rate (13 L/min) and nebulizer (30 psi), were then evaluated using the Source optimizer

Table I. MRM Parameters of the DOAs under Study

	Compound	R_t (min)	Precursor ion	Product ion 1	CE 1 (eV)	Product ion 2	CE 2 (eV)	Product ion 3	CE 3 (eV)	Fragmentor voltage (V)
1	MOR	3.55	286.1	165	40	153	40	157	40	50
2	COD	5.50	300.2	165	40	199	36	153	42	100
3	Methylephedrine	5.91	180.0	162	8	147	10	131	10	100
4	DMC	6.03	178.1	105	18	133	14	72	18	100
5	Methylone	6.36	208.0	160	16	132	20	190	10	100
6	Flephedrone	6.47	182.1	164	15	149	15	123	15	100
7	AMP	6.53	136.1	119	4	91	10	–	–	100
8	Ethcathinone	6.61	178.1	160	12	132	14	105	18	100
9	Hyoscine	6.87	304.0	156	20	138	20	121	20	100
10	6-AM	7.31	328.2	211	36	193	36	165	40	100
11	MDA	7.42	180.1	163	6	135	10	133	10	100
12	Methedrone	7.80	194.1	176	10	161	16	145	16	100
13	MAMP	7.82	150.1	119	8	91	10	–	–	100
14	Ethylone	7.95	222.1	174	18	204	12	146	18	100
15	Buphedrone	8.17	178.1	91	15	131	15	132	15	100
16	MDMA	8.78	194.1	163	10	133	15	105	15	100
17	*Oxo*-LSD	9.33	356.0	237	20	222	20	265	12	100
18	2-MMC	9.63	178.1	160	8	145	15	119	15	100
19	Butylone	9.82	222.1	174	14	204	10	191	10	100
20	Mephedrone	10.19	178.1	160	8	145	15	119	15	100
21	Norketamine	10.65	224.0	207	8	179	12	125	12	100
22	KET	11.54	238.0	125	20	220	12	207	12	125
23	BZE	11.78	290.1	168	20	105	20	82	20	125
24	4-MEC	11.92	192.1	174	10	146	16	145	18	125
25	Pentedrone	12.52	192.1	174	10	132	16	91	18	125
26	3,4-DMMC	13.58	192.1	174	10	159	15	133	15	125
27	HER	14.03	370.0	211	36	268	36	165	42	125
28	Alpha-PVP	14.23	232.2	91	22	105	25	126	25	125
29	COC	14.75	304.2	182	18	105	22	82	22	125
30	MDPV	14.84	276.2	126	25	135	25	175	20	125
31	LSD	15.53	324.2	231	24	208	26	180	26	125
32	Pyrovalerone	16.51	246.2	105	22	126	26	119	26	75
33	Bromazepam	17.37	316.0	182	30	209	30	288	30	100
34	Fentanyl	17.62	337.2	188	22	105	24	–	–	100
35	Buprenorphine	18.33	468.0	55	48	396	42	414	35	100
36	EDDP	19.03	278.0	234	30	249	20	186	30	100
37	Methadone	19.93	310.2	265	12	105	18	–	–	100
38	Lorazepam	20.42	321.0	275	20	303	10	230	20	100
39	Alprazolam	21.21	308.4	281	24	274	24	204	30	100
40	Diazepam	23.05	285.0	193	30	154	30	222	30	75

software. Once the MRM method was optimized, 10 windows of detection were created in accordance with retention times and acquisition.

SPE sorbent selection

In the present study, we tested and compared for the first time four mixed-mode cation-exchange sorbents (both weak and strong) for extracting DOAs and some of their metabolites from urine. In particular, we used one weak (Oasis WCX) and three strong (Oasis MCX, ExtraBond ECX and ExtraBond SCX) sorbents. The main differences were the stationary phase and the amount of sorbent used (150, 150, 200 and 1,000 mg, respectively). All these sorbents are based on polymeric phases, except ExtraBond SCX, which is based on a silica phase. These extraction sorbents were chosen by taking into account the characteristics of the compounds under study since most of these were basic with pK_a values ranging from 7 to 10. However, the pK_a values of benzodiazepines (BZD) are roughly 2, except for lorazepam and bromazepam, which range from 7 to 10. Some of the sorbents used have already been tested for determining synthetic cathinones (19) and ATS in urine (32), with extraction efficiencies >80% for Oasis WCX and Oasis MCX. The protocols for the two Oasis sorbents were the same as those used in the above study on synthetic cathinones because they are also basic compounds. Specifically, for Oasis MCX, the procedure was as follows: 5 mL of MeOH, 5 mL of phosphate buffer at pH 6 (conditioning), 5 mL of urine:phosphate buffer at pH 6 (loading), 2 mL MeOH (washing) and 2 mL 5% NH_4OH in MeOH (elution). For Oasis WCX, the procedure involved conditioning with MeOH and phosphate buffer at pH 7 before the sample was loaded at pH 7, washed with H_2O and eluted with MeOH (19). Because of the similar, strong characteristics between Oasis MCX and the two ExtraBond sorbents, the initial protocol for the ExtraBond sorbents was the same as for the Oasis MCX sorbents. To comparatively evaluate the four sorbents, we performed three extractions for each using pooled urine spiked at 50 ng/mL with the DOAs under study.

The two Oasis sorbents were compared first since they both have polymeric phases and were from the same manufacturer (although one was weak and the other was strong).

For greater clarity, the results are expressed as the mean $\%R_{app}$ values from the various DOA families. Specifically, the drugs were divided into opioids, cathinones, ATS, BZD and other DOAs. For Oasis WCX, the results were 31%, 46%, 19%, 11% and 19%, respectively, while for Oasis MCX, they were 47%, 82%, 78%, 26% and 69%, respectively. Better results were obtained for Oasis MCX. This strong sorbent was compared with a sorbent from a different manufacturer but with the same strong retention polymeric phase (ExtraBond ECX). Since the $\%R_{app}$ values obtained for ExtraBond ECX were the worst (19%, 35%, 29%, 14% and 37% for opioids, cathinones, ATS, BZD and other DOAs, respectively), this sorbent was discarded. Finally, another strong sorbent with a different phase based on silica (ExtraBond SCX) was evaluated and compared with the sorbent that provided the best results (i.e., Oasis MCX). Results for these two sorbents were similar. For ExtraBond SCX, they were 44%, 85%, 77%, 17% and 77%, respectively, for the above drug families. For this reason, both sorbents were chosen for further studies. All results obtained with the four sorbents are shown in Figure 1 and, as mentioned earlier, the drugs are divided into five families. For both sorbents, the lowest $\%R_{app}$ values were obtained for opioids (47% and 44%) and especially for BZDs (26% and 17%) except for two of them, i.e., lorazepam (40% and 35%) and bromazepam (38% and 40%). This different behavior for BZDs could be due to the differences between the pK_a values of these drugs and those of other compounds. A possible explanation for the low $\%R_{app}$ values obtained for the opioids could be their lower retention in the SPE sorbent, which is related to their different chemical structure compared to the other DOAs under study. To increase the low $\%R_{app}$ achieved for some compounds, we tested how the pH of the sample affected urine samples in order to increase their retention strength with the Oasis MCX and ExtraBond SCX sorbents. Specifically, pH values between 2 and 6 were tested for these sorbents by adjusting the sample pH with HCOOH ≥98%. Using different pH values showed that the results were best with ExtraBond SCX and Oasis MCX at pH 4 in both cases. This pH increased the $\%R_{app}$ of some compounds to 20% and 28% for Oasis MCX and ExtraBond SCX, respectively. For example, MOR (opioid) improved its $\%R_{app}$ at pH 4 from 21% to 41% and from 23% to 51% for Oasis MCX and ExtraBond SCX, respectively. With BZDs, the $\%R_{app}$ of alprazolam increased from 11% to 21% and from 11% to 20% for Oasis MCX and ExtraBond SCX, respectively, while the $\%R_{app}$ of diazepam increased from 8% to 16% and 9% to 18%, respectively.

Since the commercial ExtraBond SCX sorbent comprised 1,000 mg of silica phase and the Oasis MCX comprised 150 mg of polymeric phase, different amounts of Oasis MCX sorbent were tested to evaluate whether the $\%R_{app}$ values increased with a greater amount of sorbent. Specifically, we tested 150, 500 and 1,000 mg. Our results showed that 500 and 1,000 mg of Oasis MCX did not improve the $\%R_{app}$ values even when the elution was performed with 4 mL of elution solvent. Therefore, by comparing the results obtained with 150 mg of Oasis MCX to those obtained with 1,000 mg of ExtraBond SCX at pH 4, we found that, for most compounds, the results were similar for both sorbents. However, ExtraBond SCX improved the $\%R_{app}$ of some compounds with very low $\%R_{app}$ in comparison with Oasis MCX, such as KET (other DOAs), BZE (other DOAs), HER (opioid) and lorazepam (BZD). For example, KET had a $\%R_{app}$ of 7% with Oasis MCX and a $\%R_{app}$ of 44% with ExtraBond SCX, while BZE had a $\%R_{app}$ of 28% with Oasis MCX and a $\%R_{app}$ of 71% with ExtraBond SCX. On the other hand, fentanyl (opioid) and norketamine (other DOAs) improved their $\%R_{app}$ with Oasis MCX (from 32% to 47% and from 60% to 78%, respectively). As with Oasis MCX, and to improve the $\%R_{app}$, the amount of ExtraBond SCX sorbent was also studied to ascertain whether increasing the amount to 1,500 and 2,000 mg achieved better retention of the compounds. Since we studied the amount of sorbent, we also evaluated the elution volume; since the previous tests were performed with 2 mL of elution solvent, we also studied two further elutions of 1 mL (i.e., 2 + 1 + 1 mL) for 1,000, 1,500 and 2,000 mg. We concluded that, while 2 mL of elution solvent was needed for 1,000 mg, 3 mL was needed for 1,500 mg and

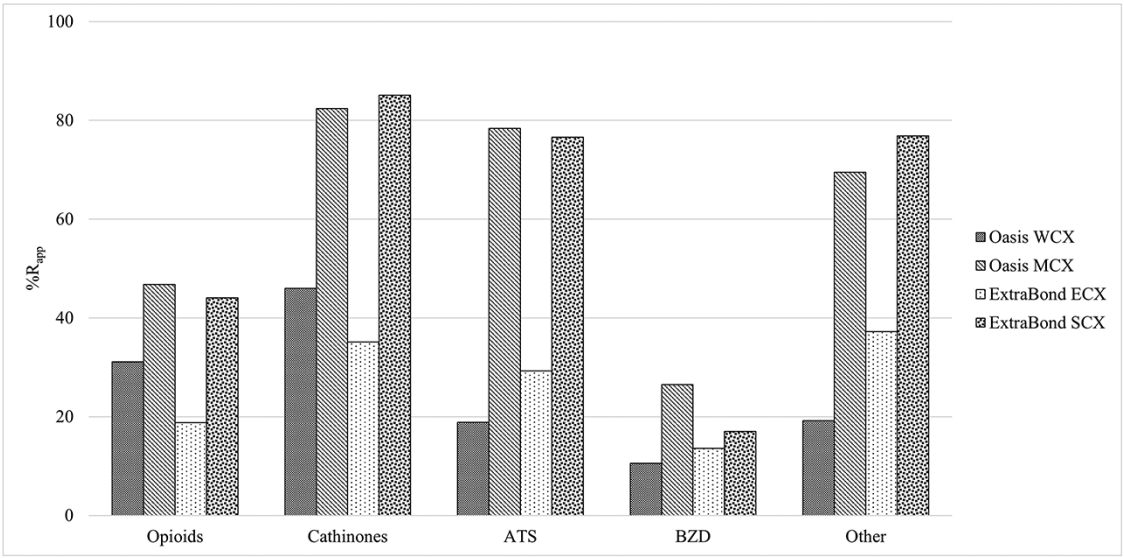

Figure 1. Recovery values for the SPE process using Oasis WCX, Oasis MCX, ExtraBond ECX and ExtraBond SCX of a urine sample spiked at 50 ng/mL with the DOAs under study.

4 mL was needed for 2,000 mg. Better $\%R_{app}$ values were obtained with 1,500 mg of sorbent (mean $\%R_{app}$ values were 39%, 72%, 67%, 26% and 63% for the opioids, cathinones, ATS, BZD and other DOAs, respectively). 1,500 mg was chosen as the optimal amount of sorbent since this generally achieved the best $\%R_{app}$, with the best results being

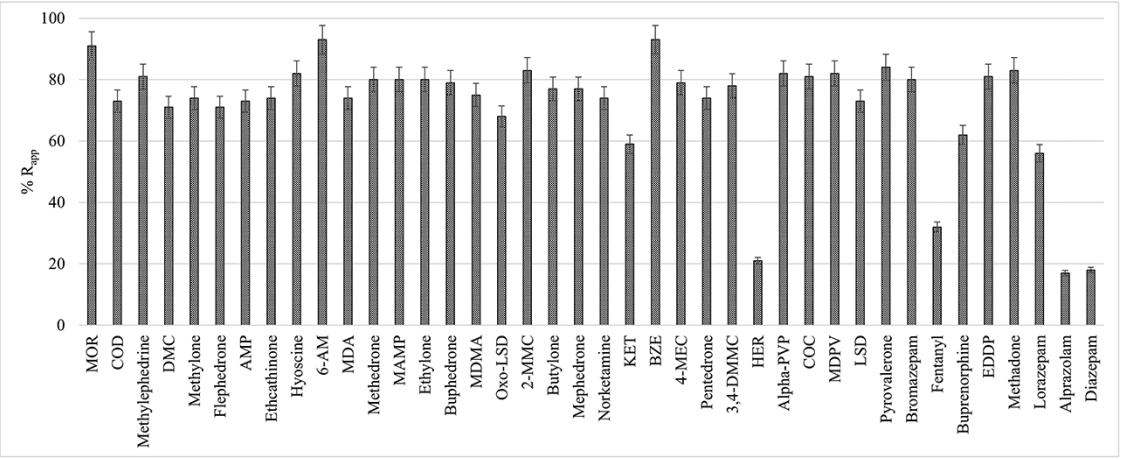

Figure 2. Recovery values of the 40 selected DOAs under the optimal SPE ExtraBond SCX conditions of a urine sample spiked at 65 ng/mL.

Table II. Validation Parameters of the DOAs under Study

					2 ng/mL		20 ng/mL		65 ng/mL	
	Compound	MDL (ng/mL)	MQL (ng/mL)	Linear range (ng/mL)	$\%R_{app}$	%ME	$\%R_{app}$	%ME	$\%R_{app}$	%ME
1	MOR	0.050	0.250	0.250–75	60	–30	83	–21	91	–10
2	COD	0.025	0.250	0.250–125	77	–22	68	–31	73	–25
3	Methylephedrine	0.015	0.250	0.250–75	69	–24	72	–32	81	–19
4	DMC	0.025	1.000	1.000–75	71	–27	53	–25	71	–29
5	Methylone	0.025	0.250	0.250–100	61	–34	68	–30	74	–25
6	Flephedrone	0.050	0.250	0.250–5 5–100	61	–26	64	–26	71	–19
7	AMP	0.025	0.250	0.250–5 5–125	65	–26	65	–26	73	–21
8	Ethcathinone	0.015	0.075	0.075–100	68	–24	67	–24	74	–22
9	Hyoscine	0.030	0.100	0.100–100	69	–28	68	–24	82	–19
10	6-AM	0.025	0.250	0.250–5 5–100	89	–14	83	–16	93	–10
11	MDA	0.050	0.250	0.250–5 5–100	70	–24	70	–25	74	–22
12	Methedrone	0.050	0.250	0.250–5 5–100	70	–25	75	–26	80	–18
13	MAMP	0.010	0.050	0.050–5 5–100	77	–23	73	–18	80	–22
14	Ethylone	0.030	0.100	0.100–5 5–100	74	–17	72	–16	80	–17
15	Buphedrone	0.025	0.250	0.250–5 5–100	79	–23	75	–25	79	–24
16	MDMA	0.035	0.100	0.100–5 5–100	73	–22	71	–21	75	–20
17	*Oxo*-LSD	0.500	1.500	1.500–100	42	–31	58	–28	68	–27
18	2-MMC	0.010	0.050	0.050–5 5–100	78	–16	79	–17	83	–15
19	Butylone	0.050	0.250	0.250–5 5–100	77	–18	72	–19	77	–15
20	Mephedrone	0.010	0.050	0.050–5 5–100	78	–21	71	–20	77	–21
21	Norketamine	0.050	0.250	0.250–5 5–100	59	–29	61	–30	74	–29
22	KET	0.010	0.050	0.050–5 5–100	75	–26	54	–30	59	–23
23	BZE	0.003	0.050	0.050–5 5–100	79	–12	89	–11	93	–14
24	4-MEC	0.003	0.250	0.250–5 5–100	57	–23	76	–23	79	–25
25	Pentedrone	0.010	0.050	0.050–5 5–100	69	–25	68	–26	74	–26
26	3,4-DMMC	0.010	0.050	0.050–75	70	–20	65	–23	78	–18
27	HER	0.100	0.500	0.500–125	18	–45	21	–42	21	–38
28	Alpha-PVP	0.003	0.050	0.050–5 5–100	82	–11	76	–14	82	–10
29	COC	0.010	0.050	0.050–5 5–75	86	–14	80	–15	81	–14
30	MDPV	0.010	0.050	0.050–5 5–75	85	–17	77	–15	82	–13
31	LSD	0.010	0.050	0.05–5 5–100	72	–22	71	–21	73	–20
32	Pyrovalerone	0.010	0.050	0.05–5 5–100	82	–15	71	–13	84	–12
33	Bromazepam	0.250	0.750	0.75–100	83	–23	75	–18	80	–24
34	Fentanyl	0.003	0.050	0.05–5 5–100	36	–15	24	–18	32	–16
35	Buprenorphine	0.010	0.075	0.075–100	47	–62	49	–49	62	–37
36	EDDP	0.003	0.050	0.05–5 5–100	77	–26	71	–28	81	–19
37	Methadone	0.010	0.050	0.05–5 5–100	77	–21	77	–17	83	–19
38	Lorazepam	0.050	0.250	0.25–5 5–100	57	–21	49	–18	56	–22
39	Alprazolam	0.250	1.000	1.00–75	19	–25	19	–19	17	–22
40	Diazepam	0.100	0.750	0.75–75	15	–23	15	–19	18	–22

obtained for BZD. This amount also improved some of the lowest $\%R_{app}$ values obtained with the other sorbents without decreasing the others. For example, COD had better results with 1,000 mg, followed by 1,500 and 2,000 mg, while methadone had better results with 2,000 mg, followed by 1,500 and 1,000 mg.

The optimal SPE procedure was therefore to use 1,500 mg of ExtraBond SCX sorbent under the following conditions: 5 mL MeOH, 5 mL H_2O at pH 4, 5 mL urine:H_2O at pH 4 (50:50, v/v), 2 mL MeOH and 3 mL 5% NH_4OH in MeOH. Then, 100 µL of 1% HCl in MeOH was added before evaporating to dryness with the MiVac. This was finally reconstituted with 500 µL of the initial mobile phase, filtered through a 0.45-µm PTFE syringe filter and injected into the LC–MS-MS. Finally, this optimal procedure was tested at a high level of concentration (65 ng/mL) in urine. Figure 2 shows the $\%R_{app}$ values of the 40 DOAs and metabolites under study. As we explained earlier, the $\%R_{app}$ values were the lowest with some opioids (HER and fentanyl) and some BZD (alprazolam and diazepam) because of their different chemical structures and different pK_a values, which make their retention strength weaker than that of the other compounds.

Method validation

The instrumental linearity of the method used in this study ranged from the IQL to 125, 200 or 250 ng/mL depending on the DOA. IDLs ranged from 0.01 to 1.5 ng/mL, and IQLs ranged from 0.1 to 5.0 ng/mL. To evaluate possible endogenous or exogenous interferences, we analyzed drug-free urine samples from laboratory staff members. The exogenous interferences studied were several cannabinoids and some sex-related drugs. No interferences were observed at the same retention times of the DOAs under study.

The method validation parameters are given in Table II, which shows that the calibration model was adjusted to one or two matrix-matched calibration curves from the MQL to 75, 100 or 125 ng/mL depending on the DOA under study, with r^2 of >0.99 for all compounds. The MDLs of the method ranged from 0.003 to 0.500 ng/mL, while the MQLs ranged from 0.050 to 1.500 ng/mL. $\%R_{app}$, ME, repeatability and reproducibility were studied at three levels of concentration in urine: low (2 ng/mL), medium (20 ng/mL) and high (65 ng/mL). $\%R_{app}$ values ranged from 42% to 89% (2 ng/mL), from 49% to 89% (20 ng/mL) and from 56% to 93% (65 ng/mL) for these three levels. For %ME, these values ranged from –62% to –11%, from –49% to –11% and from –37% to –10%, for low, medium and high concentration levels, respectively. For repeatability and reproducibility, values <20% were achieved for all three concentration levels. The stability of the DOAs was also evaluated at 10°C for 50 h. Any evidence of degradation was observed during this period with RSD values <8% for the three calibration levels by injecting each calibrator every 10 h, as it was previously tested by Glickberg et al. (33) for some synthetic cathinones in urine.

Finally, the method was applied to urine blind samples spiked at three concentrations, i.e., 7, 45 and 60 ng/mL. Accuracy was calculated as the error between the concentration calculated with the matrix-matched calibration curve and the concentration spiked in the same sample. The values obtained ranged from 8% to 17% for 7 ng/mL, from 9% to 15% for 45 ng/mL and from 5% to 11% for 60 ng/mL.

This method achieves low MDLs and MQLs and determines the above compounds at the usual levels at which they

Table III. Compounds Detected in Urine Samples from Women Starting a Detoxification Program (n = 3)

Specimen	Analyte detected	Concentration found (ng/mL)	BZE/COC
1	Lorazepam	0.531 ± 0.020	
	Diazepam	144 ± 3	
2	COC	2.11 ± 0.06	7.4
	BZE	15.6 ± 0.4	
	HER	2.64 ± 0.06	
3	COC	0.48 ± 0.06	37.9
	BZE	18.2 ± 0.4	
4	COC	<MQL	
	BZE	7.64 ± 0.11	
5	BZE	4.56 ± 0.07	
	Lorazepam	4.73 ± 0.03	
6	COC	4.90 ± 0.09	406.7
	BZE	1993 ± 17	
7	COC	2.97 ± 0.07	11.1
	BZE	54.1 ± 0.4	
8	COC	<MQL	
	BZE	0.39 ± 0.05	
	Lorazepam	13.71 ± 0.08	
	Diazepam	5.3 ± 0.3	
9	–		
10	COC	7.72 ± 0.14	1.1
	BZE	8.8 ± 0.1	
11	COC	<MQL	
	BZE	42.4 ± 0.4	
12	COC	119 ± 2	2.2
	BZE	257.8 ± 0.4	
	Diazepam	2.21 ± 0.09	
13	HER	58.3 ± 0.8	37.4
	MOR	1.78 ± 0.11	
	6-AM	9.4 ± 0.3	
	COC	6.34 ± 0.10	
	BZE	235.5 ± 0.4	
	Diazepam	10.3 ± 0.3	
14	BZE	1.69 ± 0.04	
	HER	<MQL	
	Bromazepam	3.74 ± 0.14	
15	COC	1.94 ± 0.11	57.9
	BZE	112.4 ± 0.4	
	HER	6.5 ± 0.3	
16	COC	1.31 ± 0.09	36.8
	BZE	47.8 ± 0.4	
	HER	30.9 ± 0.8	
	Lorazepam	3.03 ± 0.03	
17	COC	10.9 ± 0.3	23.7
	BZE	257.9 ± 0.4	
	Diazepam	2.8 ± 0.2	
18	COC	535 ± 2	17.9
	BZE	9572 ± 1	
	HER	6.5 ± 0.3	
	MDMA	1.52 ± 0.03	
	MDA	<MQL	
19	BZE	22.2 ± 0.4	
	HER	6.5 ± 0.3	
	Diazepam	12.1 ± 0.4	
20	COC	<MQL	
	BZE	24.4 ± 0.4	
	Diazepam	15.1 ± 0.5	
21	COC	14.5 ± 0.6	4.2
	BZE	60.4 ± 0.4	
	Fentanyl	42.2 ± 0.7	
22	–		

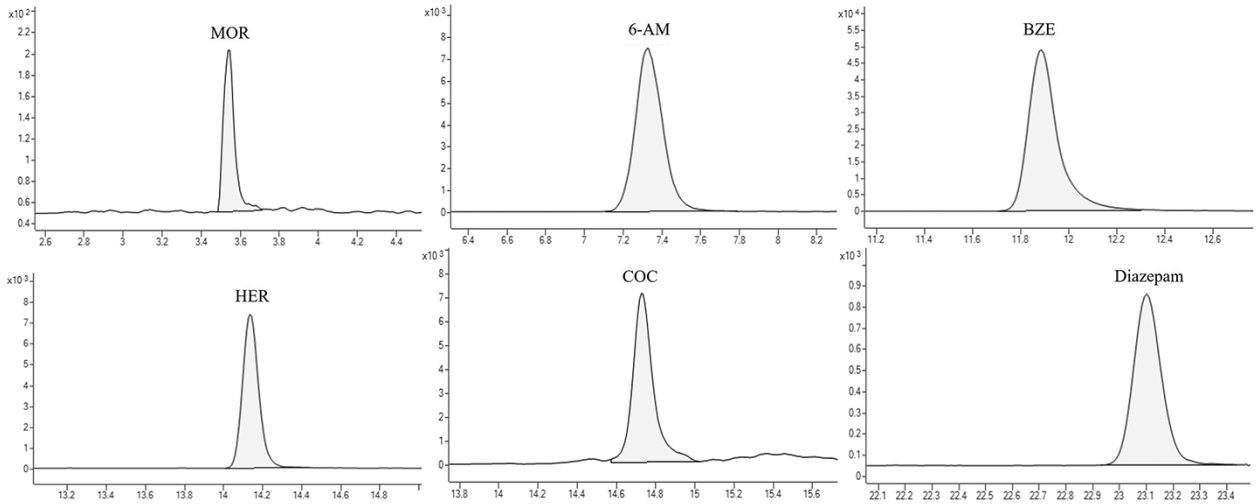

Figure 3. Chromatogram of the analyzed Specimen 13.

are present in the urine of drug users. Since it includes some of the classic DOAs that are most consumed, as well as some of the new synthetic drugs currently available on the market and some of their metabolites, it is a useful method for toxicological and forensic laboratories.

Application to real cases

Twenty-two urine samples from women in a detoxification program were analyzed using the present method. Of these samples, 91% were found to be positive for one or more DOAs (20 out of 22 samples). COC, BZE, HER and diazepam were the substances most detected. Detailed results for each sample analyzed are shown in Table III: COC was found in 16 specimens, its metabolite BZE was found in 19, diazepam was found in 7, lorazepam was found in 4, HER was found in 7, MOR and 6-AM were found in 1, bromazepam was found in 1, MDMA and MDA were found in 1 and fentanyl was found in 1. Multiple drug abuse was identified in 14 samples, mainly involving the consumption of COC as well as some BZDs, which were the second most common group found in urine samples, followed by HER. Once the samples were analyzed, the mean concentration for each DOA was calculated ($n = 3$). Then, the uncertainty of each compound was determined with 95% degree of confidence.

Concentrations of COC ranged from <MQL to 535 ng/mL, while the concentration of BZE ranged from 0.32 to 9,572 ng/mL. The concentration of HER ranged from <MQL to 58.3 ng/mL, while MOR and 6-AM were found at 1.78 and 9.4 ng/mL, respectively. The concentrations of lorazepam and diazepam in urine ranged from 0.531 to 13.71 ng/mL and from 2.21 to 144 ng/mL, respectively, while bromazepam was found at 3.74 ng/mL. Finally, MDMA and MDA were found in Specimen 18 at 1.52 ng/mL and <MQL, respectively. When the concentrations were found to be outside the linearity range, the samples were diluted to interpolate the results in the matrix-matched calibration curves, and the dilution factor was applied to obtain the real concentrations in the urine samples. An example of a chromatogram of Sample 13, in which MOR, 6-AM, BZE, COC, HER and diazepam were identified, is shown in Figure 3.

Table III also shows the ratios between the concentrations of BZE and COC. Although these ratios depend on the metabolism of each individual and the time the drug was consumed, low ratios generally mean that COC is not or is hardly metabolized to BZE (recent drug intake), while high ratios indicate that it is strongly metabolized (long-term drug intake). For example, Specimen 3 has the highest BZE/COC ratio, which means that most of the COC has been metabolized to BZE and tentatively indicates that consumption was long term. On the other hand, Specimen 10 has the lowest ratio, which may indicate recent drug intake since COC has not been completely metabolized to BZE. In the case of Specimen 5, for example, the BZE/COC ratio could not be calculated as COC could not be quantified, perhaps because consumption occurred a long time ago.

Conclusions

A method using SPE followed by LC–MS-MS to determine a group of DOAs (both traditional and new) in urine has been successfully developed. This method performed excellently for the simultaneous screening of 40 DOAs comprising several families and some metabolites. The ability to simultaneously determine a large number of drugs enables high-throughput screening and considerably reduces analysis runtimes. Method optimization in which four SPE sorbents were tested and compared for all compounds found that the best results were obtained with ExtraBond SCX. This is the first time that this commercial sorbent has been used for extracting a large number of DOAs from urine. The resulting method is capable of achieving very low MDLs and MQLs and is suitable for identifying these compounds in the urine of drug users. To prove the applicability of the method, we used it to successfully analyze authentic urine samples from women following a detoxification program. We found that COC was the most consumed drug, and it was confirmed since BZE was also found. This method has therefore proved suitable for toxicological and forensic analysis and for monitoring individuals under a detoxification program. It has also demonstrated

the importance of simultaneously identifying and quantifying multiple DOAs since it can discriminate between various DOAs in toxicological analyses assessing polyconsumption.

Acknowledgments

We would like to thank the volunteers from the Fundació CECAS in Tarragona for providing samples.

Funding

We would like to thank the Spanish Ministerio de Ciencia, Innovaci'on y Universidades, the Fons Social Europeu, the Iniciativa d'Ocupació Juvenil EJ2018-003102-A) and the European Regional Development Fund (ERDF) (Project: PID2020-114587GB-I00) for their financial support.

Ethical approval

All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

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