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Identification and Measurement of Illicit Drugs and Their Metabolites in Urban Wastewater by Liquid Chromatography−Tandem Mass Spectrometry

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Residues of illicit drugs and their metabolites that are excreted by humans may flow into and through wastewater treatment plants. The aim of this study was to develop a method for the determination of cocaine, amphetamines, morphine, cannabinoids, methadone, and some of their metabolites in wastewater. Composite 24-h samples from urban treatment plants were enriched with deuterated internal standards before solid-phase extraction. High-pressure liquid chromatography tandem mass spectrometry with multiple reaction monitoring was used for quantitation. Recoveries were generally higher than 80%, and limits of quantifications were in the low nanograms-per-liter range for untreated and treated wastewater. The overall variability of the method was lower than 10% for untreated and 5% for treated wastewater. The method was applied to wastewater samples coming from two treatment plants in Italy and Switzerland. Quantification ranges were found to be 0.2−1 μg/L for cocaine and its metabolite benzoylecgonine, 80−200 ng/L for morphine, 10 ng/L for 6-acetylmorphine, 60−90 ng/L for 11-nor-9-carboxy-Δ9-tetrahydrocannabinol, 10−90 ng/L for methadone and its main metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, and lower than 20 ng/L for amphetamines. As previously reported for cocaine, this method could be useful to estimate and monitor drug consumption in the population in real time, helping social scientists and authorities to combat drug abuse.

The use of illicit drugs is increasing worldwide, and millions of individuals are reported to be current users of cocaine, heroin, amphetamine-like stimulants, marijuana, and other drugs, with significant consequences for human health and social behavior.[1]

Several methods have been developed to diagnose illicit drug consumption in clinical and forensic toxicology and to test for doping.[2] Different matrixes have been tested for markers of drug abuse.[3] Solid phase (SPE)[4] and headspace solid-phase dynamic[5] methods have been used to extract residues and metabolites of illicit drugs from biological fluids and hair. Specific analytical methods have been set up for their measurement in urine, sweat,[3−6] blood,[7] saliva,[8,9] and hair.[10] Gas chromatography−mass spectrometry (GC/MS)[11] and liquid chromatography−mass spectrometry (LC−MS)[12] are the techniques of choice for quantitative analysis. GC/MS with electron impact or positive ion chemical ionization and MS−MS fragmentation[13,14] has been used to analyze simultaneously up to 30 different illicit drugs in oral fluids.[15,16] Liquid chromatography coupled to atmospheric pressure chemical ionization and tandem mass spectrometry (LC−APCI-MS−MS)[17−20] has been used to measure amphetamines, opioids, cocaine and metabolites, methadone, and hypnotics.

Despite the availability of these analytical tools, there are still difficulties in estimating the real magnitude of illicit drug con-

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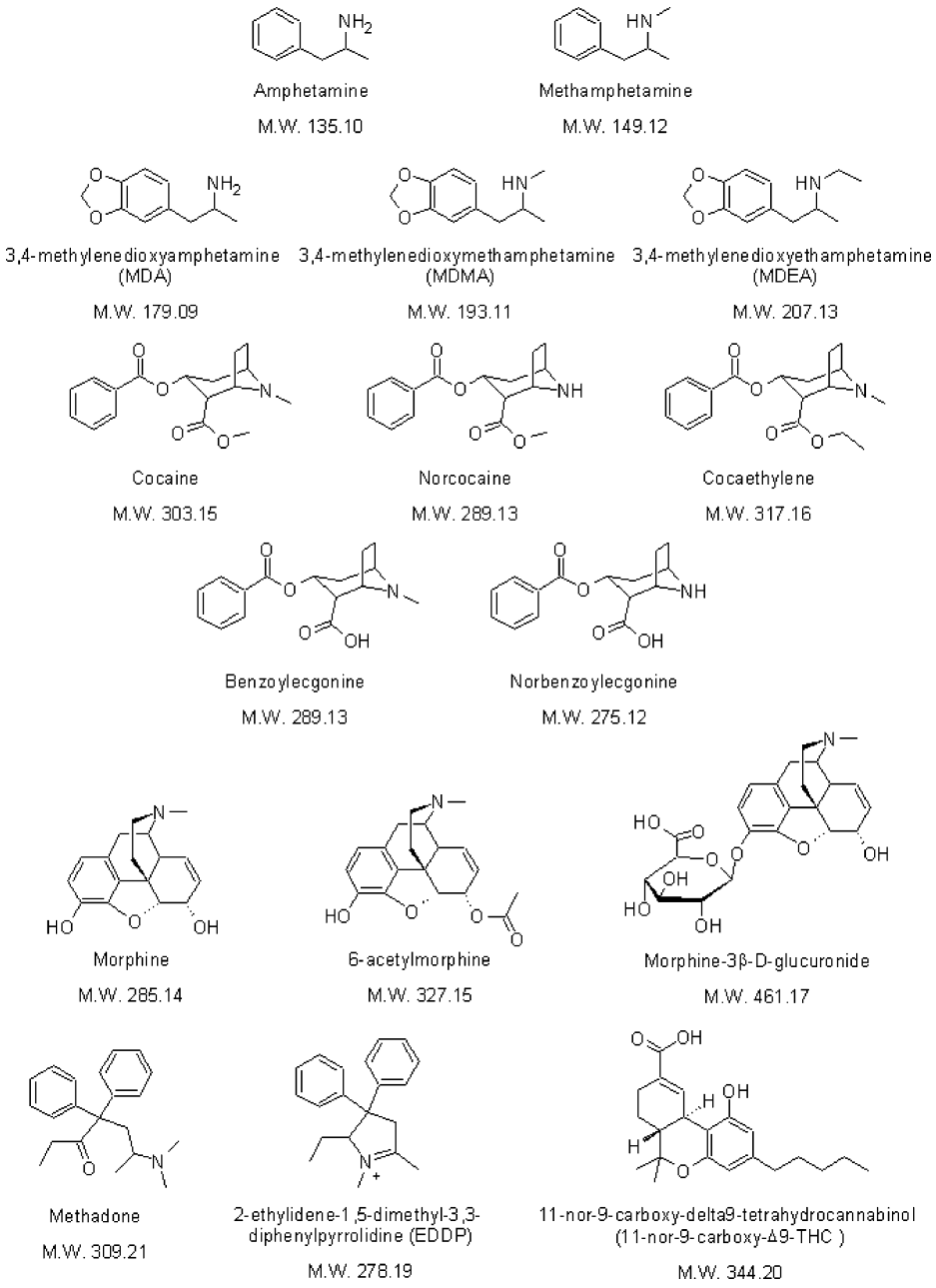

Figure 1. Structures and molecular weights of illicit drugs and their metabolites.

sumption in the population. Specific analysis of biological fluids may discover drug consumption by individuals, but estimates for a community are based on indirect methods, such as population surveys, crime statistics, and consumer interviews,[21,22] which might be unreliable.

A first study to estimate cocaine consumption directly in the community was recently published by our group.[23] Levels of cocaine and its metabolite benzoylecgonine in surface and wastewater were used to calculate environmental loads and to estimate cocaine consumption in different communities. This paper received worldwide media coverage and appreciation from the scientific press,[24,25] suggesting widespread interest in this novel approach.

We have now applied our analytical method to other illicit drugs, such as amphetamines, morphine, cannabinoids, and methadone. Residues and metabolites were measured in wastewater from treatment plants in Milan, Italy, and in Lugano, Switzerland. The analytical method is derived from a previous method developed by our group to measure human and veterinary

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Table 1. Analytical Grouping of the Substances Under Analysis, with the Instrumental Conditions and Chromatographic Retention Times

group	substance	retention time (min)	HPLC method	MS method
amphetamines	amphetamine	9.15	column, XTerra MS C18, 100 × 2.1 mm, 3.5 μm; flow, 200 μL/min, injection loop 20 μL; solvent A, acetic acid 0.05% in water; solvent B, acetonitrile; gradient, from 0 to 24% of B in 14 min, then to 100% B in 2 min (2-min hold) and to 0% B in 2 min (8-min hold for reequilibration).	positive ion IonSpray-MRM (Table 2)
	amphetamine-d_6	9.12		
	methamphetamine	9.79		
	methamphetamine-d_9	9.72		
	MDA	9.94		
	MDA-d_5	9.90		
	MDMA	10.44		
	MDMA-d_5	10.40		
	MDEA	11.26		
	MDEA-d_5	11.24		
cocaine and metabolites	cocaine	14.93	same conditions as amphetamines, except gradient from 0 to 30% of B in 18 min, then to 100% B in 2 min (2-min hold) and to 0% B in 2 min (8-min hold for reequilibration)	positive ion IonSpray-MRM (Table 2)
	cocaine-d_3	14.90		
	norcocaine	15.49		
	norcocaine-d_3	15.46		
	cocaethylene	16.98		
	cocaethylene-d_8	16.92		
	benzoylecgonine	14.33		
	benzoylecgonine-d_3	14.30		
	norbenzoylecgonine	14.05		
morphine and metabolites, methadone and metabolites	morphine	7.41	same conditions as amphetamines, except gradient from 0 to 40% of B in 22 min, then to 100% B in 2 min (2-min hold) and to 0% B in 2 min (8-min hold for reequilibration)	positive ion IonSpray-MRM (Table 2)
	morphine-3β-D-glucuronide	7.32		
	morphine-3β-D-glucuronide-d_3	7.31		
	6-acetylmorphine	10.90		
	6-acetylmorphine-d_6	10.86		
	methadone	20.86		
	metadone-d_3	20.84		
	EDDP	19.15		
	EDDP-d_3	19.12		
Δ9-tetrahydro-cannabinol metabolites	11-nor−9-carboxy-Δ9-THC	10.60	same conditions as amphetamines, except solvent A, triethylamine 0.05% in water; gradient from 0 to 45% B in 12 min, then to 100% B in 2 min (2-min hold) and to 0% B in 2 min (8-min hold for reequilibration).	negative ion IonSpray-MRM (Table 2)
	11-nor−9-carboxy-Δ9-THC−d_3	10.58		

pharmaceuticals in surface and wastewater[26,27] and is based on SPE and high-pressure liquid chromatography tandem mass spectrometry (HPLC−MS−MS).

This is the first application of LC−MS−MS methods to the simultaneous analysis of a wide variety of illicit drugs in wastewater. Methamphetamine, MDMA, and cocaine have been detected previously in treated wastewater,[28] raw wastewater, and rivers.[23] As previously proposed,[29] this approach might be useful for estimating and monitoring drug consumption in the population in real time, thus providing a new tool to challenge and combat drug abuse.

EXPERIMENTAL SECTION

Chemicals and Materials. Illicit drugs and metabolites analyzed were the following: cocaine and its metabolites benzoylecgonine, norbenzoylecgonine, norcocaine, and cocaethylene; amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA, or ecstasy), and 3,4-methylenedioxyethamphetamine (MDEA); morphine, morphine-3β-D-glucuronide, 6-acetylmorphine (a metabolite of heroin); 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (11-nor-9-carboxy-Δ9-THC), the main metabolite of Δ9-tetrahydrocannabinol; and methadone and its main metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP). Their molecular structures are shown in Figure 1.

The following deuterated molecules were used as internal standards (IS): cocaine-d_3, benzoylecgonine-d_3, norcocaine-d_3, cocaethylene-d_8, amphetamine-d_6, methamphetamine-d_9, MDA-d_5, MDMA-d_5, MDEA-d_5, morphine-d_3, morphine-3β-D-glucuronide-d_3, 6-acetylmorphine-d_6, methadone-d_3, EDDP-d_3, and 11-nor-9-carboxy-Δ9-THC-d_3.

Reference standards of amphetamine, methamphetamine, amphetamine-d_6, and methamphetamine-d_9 were acquired from Salars SpA (Como, Italy). The other standards were acquired from Cambridge Isotope Laboratories (Andover, MA). The standards, available as solutions in methanol or acetonitrile (1 or 0.1 mg/mL), were diluted up to 10 ng/μL with methanol and stored at −20 °C in the dark. Working solutions containing all the substances to be analyzed were prepared before each analytical run.

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Table 2. Optimum MS Source and Analyzer Conditions for MRM Determination of Illicit Drugs and Their Metabolites[a]

substance	voltage (V) orifice	voltage (V) ring	precursor ion (m/z)	product ion I (*m/z*) and collision energy (eV)	product ion II (*m/z*) and collision energy (eV)
amphetamine	32	150	136.1	119.1 (−14)	91.05 (−24)
amphetamine-d_6			142.1	125.1	93.05
methamphetamine	30	140	150.1	119.1 (−16)	91.05 (−26)
methamphetamine-d_9			159.1	125.1	93.05
MDA	26	140	180.1	133.1 (−24)	105.1 (−20)
MDA-d_5			185.1	138.1	110.1
MDMA	30	140	194.1	133.1 (−30)	105.1 (−32)
MDMA-d_5			199.1	135.1	107.1
MDEA	36	180	208.1	133.1 (−28)	105.1 (−36)
MDEA-d_5			213.1		
cocaine	40	200	304.2	182.1 (−28)	105.1 (−42)
cocaine-d_3			307.2	185.1	
norcocaine	44	240	290.1	168.1 (−24)	136.1 (−32)
norcocaine-d_3			293.1	171.1	
cocaethylene	50	240	318.2	196.1 (−28)	82.1 (−44)
cocaethylene-d_8			326.2	204.1	85.1
benzoylecgonine	44	240	290.1	168.1 (−24)	105.1 (−42)
benzoylecgonine-d_3			293.1	171.1	
norbenzoylecgonine	40	240	276.1	154.1 (−24)	136.1 (−30)
morphine	56	260	286.1	201.1 (−36)	165.1 (−54)
morphine-d_3			289.1		
morphine−3β-D-glucuronide	56	260	462.2	286.2 (−40)	201.1 (−60)
morphine−3β-D-glucuronide-d_3			465.2	289.2	
6-acetylmorphine	50	260	328.2	211.1 (−36)	165.1 (−50)
6-acetylmorphine-d_6			334.2		
methadone	44	240	310.2	265.1 (−22)	105.1 (−36)
methadone-d_3			313.2	268.1	
EDDP	50	260	278.1	234.1 (−42)	186.1 (−48)
EDDP-d_3			281.1		189.1
11-nor−9-carboxy-Δ9-THC	−64	−260	343.1	299.1 (30)	245.1 (40)
11-nor−9-carboxy-Δ9-THC-d_3			346.1	302.1	248.1

[a] For the deuterated internal standards, only differences from the corresponding unlabeled substances are shown.

Methanol for pesticide analysis and ammonium hydroxide solution RPE (30%) were acquired from Carlo Erba (Italy); acetonitrile for LC−MS, from Riedel de Haen (Seelze, Germany); hydrochloric acid (37%) and triethylamine (TEA), from Merck (Darmstadt, Germany); and acetic acid, from Fluka (Buchs, Switzerland). HPLC grade Milli-Q water was obtained with a Milli-RO Plus 90 apparatus (Millipore, Molsheim, France). Cartridges for solid-phase extraction (3-mL disposable Oasis MCX, 60 mg) and the analytical HPLC column (XTerra MS C18, 100 × 2.1 mm, 3.5 μm) were acquired from Waters Corp., Milford, MA.

Sample Collection. Samples were collected from two wastewater treatment plants (WWTPs), one in Milan-Nosedo (Italy) and one in Lugano (Switzerland). The Nosedo plant processes wastewater from the central and eastern areas of the city of Milan by means of two sewers; the Lugano plant collects wastewater from the entire metropolitan area. The first plant serves a population of 1 250 000 with a mean flow rate of 380 000 m^3/day, the second serves 120 000 people with a mean flow rate of 60 000 m^3/day. Both plants treat domestic wastewater primarily and are equipped with pretreatment and primary and secondary treatment facilities, that is, primary settling and activated sludge processes.

For each plant, two 24-h composite samples, each obtained by pooling water collected every 20 min by automatic sampling devices, were collected from the influent (untreated) and from the effluent (treated wastewater) every day for eight consecutive days. Samples were collected in dry weather during February and March 2006. Water samples (1−2 L each) were stored in dark glass bottles at 4 °C for a maximum of 3 days, then extracted and analyzed. Samples were filtered on a glass microfiber filter GF/A 1.6 μm (Whatman, Kent, U.K.) prior to extraction.

Solid-Phase Extraction. Solid-phase extraction of illicit drugs and their metabolites was performed using mixed reversed-phase/cation-exchange cartridges (Oasis-MCX), similar to a method developed for the analysis of pharmaceuticals in wastewater.[27] Briefly, wastewater samples (50 mL) were spiked with 20 ng of each IS and the pH was adjusted to 2.0 with 37% HCl. The Oasis MCX cartridges were conditioned before use by washing with 6 mL of methanol, 3 mL of Milli-Q water, and 3 mL of water acidified to pH 2. Samples were then passed through the cartridges under vacuum at a flow rate of 10 mL/min. Cartridges were vacuum-dried for 5 min and eluted with 3 mL of methanol and 3 mL of a 2% ammonia solution in methanol. The eluates were pooled and dried under a nitrogen stream.

Liquid Chromatography−Tandem Mass Spectrometry (HPLC−MS−MS). Dried samples were redissolved in 200 μL of Milli-Q water, centrifuged, and transferred into glass vials for instrumental analysis. The HPLC system consisted of two Series 200 pumps and a Series 200 auto sampler (Perkin-Elmer, Norwalk, CT). The MS system was an API 3000 triple quadrupole mass spectrometer equipped with a turbo IonSpray source (Applied Biosystems−Sciex, Thornhill, Ontario, Canada).

Substances to be analyzed were divided into four groups to optimize chromatographic separation and to allow mass spectrometric detection of at least two fragment ions for each compound.

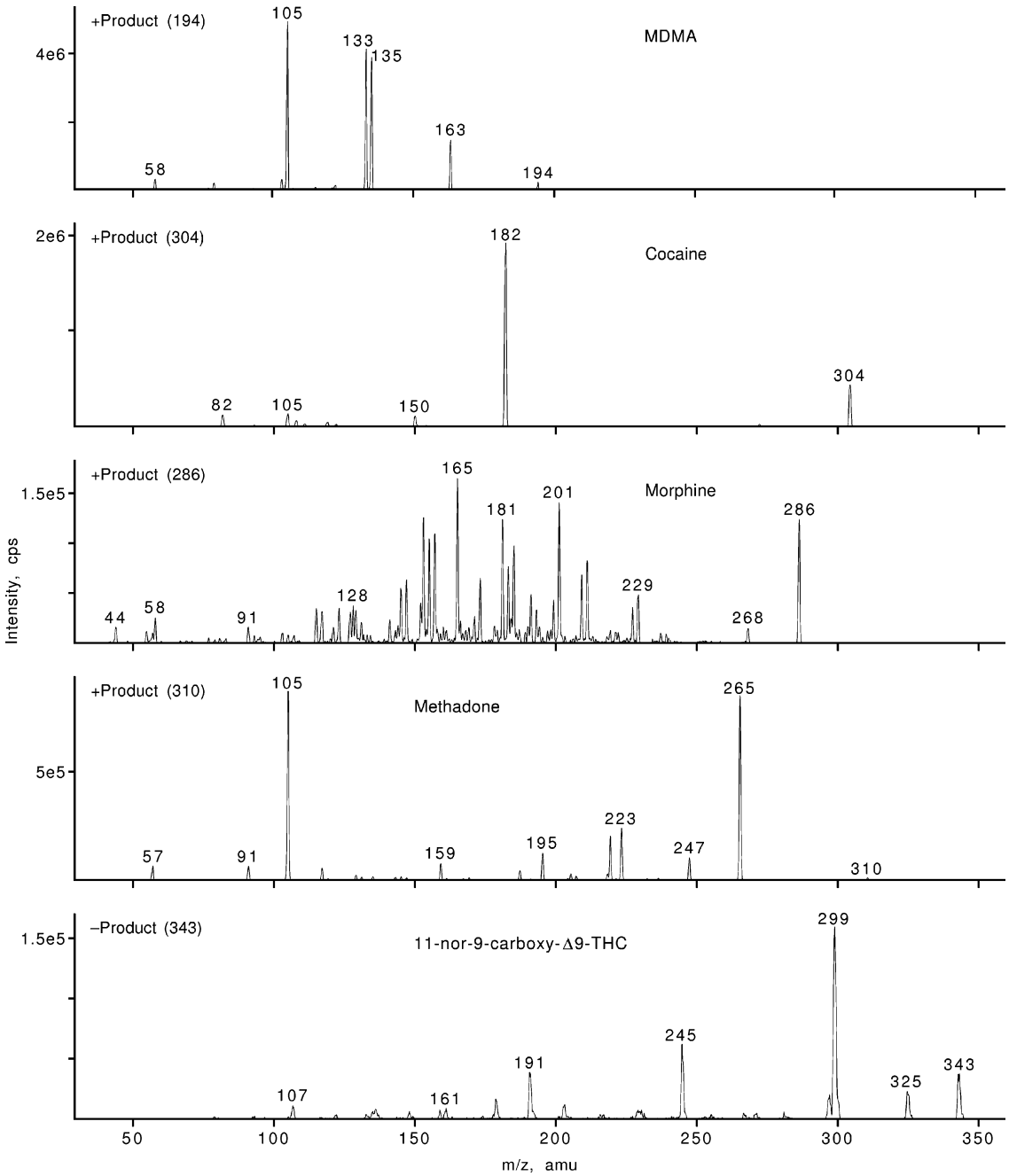

Figure 2. CID mass spectra for some of the drugs and metabolites analyzed (positive ions for MDMA, cocaine, morphine, and methadone; negative ions for 11-nor-9-carboxy-Δ9-THC). Instrumental conditions are shown in Tables 1 and 2.

The composition of the groups and the relative instrumental conditions are shown in Table 1.

The mobile phase was directly introduced into the ion source, which was operated with a turbo ion gas temperature of 350 °C. Mass spectrometric analyses were performed in multiple reaction monitoring (MRM) mode, measuring the fragmentation products of the protonated or deprotonated pseudomolecular ions of each substance and each deuterated analogue (Table 2). The choice of fragmentation products for each analyte and the optimization of collision-induced dissociation (CID) energies and other instrumental parameters were done in continuous-flow mode using standard solutions at concentrations of ~1 ng/μL. Figure 2 shows example CID spectra for some of the drugs or metabolites that were analyzed.

Quantification and Method Validation. Each compound was quantified by MRM using the two most abundant precursor/product ion transitions. Retention times were also compared with reference standards to identify the compounds. Each drug was quantified by isotope dilution using its corresponding deuterated analogue as IS (except for norbenzoylecgonine, which was quantified using benzoylecgonine-d_3).

Six-point calibration curves were generated by injecting standard solutions with different amounts of each substance (0, 2, 6, 20, 60, 200 ng) and a fixed amount of each IS (20 ng). The first point was also used as an instrumental blank.

Instrumental detection limits (IDL) and instrumental quantification limits (IQL) were determined by direct injection of increasing amounts of each substance, starting with low picogram

Table 3. Recoveries of Illicit Drugs and Their Metabolites (Mean ± SD), Instrumental Limits of Quantification (IQL) and Whole-Method Limits of Quantification (LOQ) in WWTP Influents and Effluents

	recovery + SD (%)			LOQ (ng/L)	
illicit drug	influent	STP effluent	IQL (pg/ injected)	influent	effluent
benzoylecgonine	107 ± 8.9	107 ± 2.3	15	1.98	0.92
norbenzoylecgonine	85 ± 4.7	104 ± 2.5	13	0.94	0.56
cocaine	96 ± 4.8	94 ± 3.6	18	1.4	0.99
norcocaine	112 ± 6.9	119 ± 2.0	23	1.92	0.67
cocaethylene	109 ± 4	98 ± 2.3	52	0.95	0.66
morphine	88 ± 6.6	75 ± 3.4	250	3.95	3.2
6-acetylmorphine	106 ± 4.5	93 ± 7.6	230	5.3	3.08
morphine−3β-D-glucuronide	90 ± 10.5	67 ± 0.1	12	0.63	0.48
amphetamine	110 ± 4.5	103 ± 4.2	380	5.4	2.8
methamphetamine	112 ± 6.5	97 ± 3.4	208	3.7	1.11
MDA	102 ± 3.3	98 ± 3.1	530	8.7	2.6
MDMA	104 ± 2.3	97 ± 3.2	278	6.3	1.74
MDEA	107 ± 3.7	89 ± 3.3	280	4.19	1.64
methadone	105 ± 3.1	90 ± 2.0	120	1.14	0.97
EDDP	88 ± 3.2	85 ± 4.3	103	1.64	0.6
11-nor−9-carboxy-Δ9-THC	51 ± 1.3	61 ± 4.0	307	1.75	0.94

quantities of substance. The detection limits (LOD) and quantification limits (LOQ) for the whole method were calculated by spiking wastewater samples with different amounts of the substances: 50 mL of a WWTP influent were spiked with 0.5−5 μg/L and 50 mL of a WWTP effluent with 0.1−0.5 μg/L of each substance, depending on the typical concentrations in such samples. The IDL and LOD were calculated as the concentrations giving peaks for which the signal-to-noise ratio was 3, and IQL and LOQ were calculated as the concentrations giving peaks for which the signal-to-noise ratio was 10.

The recoveries and the repeatability of the analytical method were tested by analyzing influent and effluent samples (50 mL) in triplicate. Influent samples were spiked with 5 μg/L of benzoylecgonine; 2 μg/L of cocaine, morphine, EDDP, and 11-nor-9-carboxy-Δ9-THC; and 0.5 μg/L of the other drugs before extraction and with 0.4 μg/L of IS after extraction. Effluent samples were spiked with 0.5 μg/L of benzoylecgonine, cocaine, and morphine and 0.1 μg/L of the other drugs before extraction and with 0.2 μg/L of IS after extraction. Blanks were analyzed throughout the course of these determinations to test for and correct bias.

The instrumental repeatability and precision were assessed by triplicate injections of two standard mixtures (0.1 and 1 ng/μL, 5 μL injected). The instrumental variability was measured for wastewater by running three replicates of an extracted sample. The linearity of the calibration curves was tested for concentration ranges that are normally measured in wastewater. Eight pooled solutions with concentrations between 0.1 and 1000 ng of each drug were prepared from standard mixtures in 100 μL of Milli-Q water. The instrumental repeatability was assessed on the calibration curves by injecting the standard solutions (0.1 and 1 ng/μL) three times in the same day and on different days. The intra- and interday correlation factors (r^2) were calculated.

Stability in Wastewater. The stability of the illicit drugs and their metabolites in wastewater was investigated by analyzing solutions stored in dark glass bottles for 3 days. The stability tests were run in triplicate using influent wastewater under the same conditions in which wastewater samples were normally stored. The samples (50 mL) were spiked with 5 μg/L of benzoylecgonine; 2 μg/L of cocaine, morphine, EDDP, and 11-nor-9-carboxy-Δ9-THC; and 0.5 μg/L of other drugs and were stored for three days at 4 °C. Another 50-mL sample was used as the control (no drugs added) and processed in the same way. The analyses were carried out immediately after spiking and after 3 days of incubation. Quantitative results were obtained by adding 20 ng of IS before extraction, and the residual amounts of drugs or metabolites were calculated by subtracting the amounts already present in the control sample.

RESULTS AND DISCUSSION

Solid-Phase Extraction. The recoveries (and standard deviations) obtained by spiking WWTP influents and effluents with various amounts of compounds are listed in Table 3. Extractions were performed with an Oasis MCX, a mixed reversed-phase/cation exchange cartridge that is stable at low pH. The cartridge can retain all compounds studied here because it has two phases.

Recoveries in WWTP influents and effluents did not differ substantially and were generally >80%, except for 11-nor-9-carboxy-Δ9-THC (51%) in influents and morphine (75%), morphine-3β-D-glucuronide (67%), and 11-nor-9-carboxy-Δ9-THC (61%) in effluents. Standard deviations were 10% or lower in influents and generally 5% or lower in effluents. For norbenzoylecgonine and 11-nor-9-carboxy-Δ9-THC, recoveries were lower in influents than in effluents, probably because of matrix effects.

Liquid Chromatography−Mass Spectrometry. As described above, the substances analyzed were divided into four groups (Table 1) for the purpose of simultaneous analysis based on their chromatographic and ionization characteristics. One group consisted of 11-nor-9-carboxy-Δ9-THC and 11-nor-9-carboxy-Δ9-THC-d_3, which were analyzed in the negative ion mode. Three other groups of substances were analyzed in the positive ion mode under chromatographic conditions tailored to optimize separation and sensitivity for each component. The large number of MRM transitions to be monitored (56) would not allow the simultaneous detection of all the substances with a sufficiently short cycle time. Typical chromatograms obtained from the analysis of wastewater are shown in Figure 3.

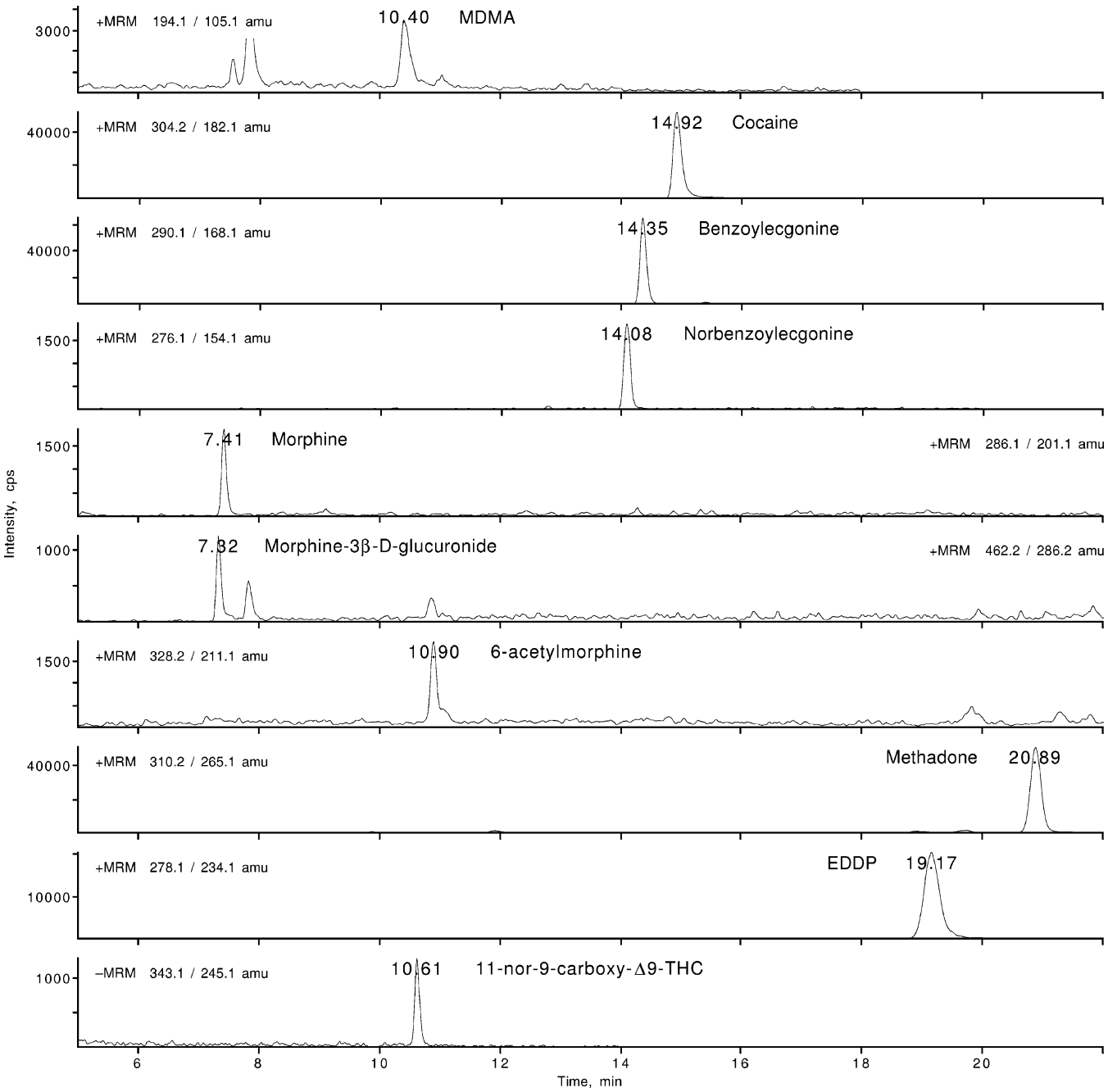

Figure 3. HPLC-MRM chromatograms of the most abundant illicit drugs and their metabolites in untreated wastewater. Instrumental conditions are shown in Tables 1 and 2.

The use of deuterated analogues for each substance (except for norbenzoylecgonine) was helpful for minimizing the matrix effects that can affect electrospray sources when analyzing real samples. Since the ions of each substance and its corresponding deuterated analogue are chemically equivalent and the retention times differ only minimally, suppression phenomena will affect both in a similar way.

Quantification and Method Validation. Table 3 reports IQL and LOQ values for influents and effluents for the substances under analysis. IQLs were lower than 300 pg/injected, except for amphetamine (380 pg/injected) and MDA (530 pg/injected). LOQs for influents were lower than 2 ng/L, except for morphine, 6-acetylmorphine (4 and 5 ng/L, respectively), and amphetamines (3.7−8.7 ng/L). LOQs for effluents were lower than 1 ng/L, except for morphine, 6-acetylmorphine (3 ng/L), and amphetamines (1.0−2.8 ng/L). A possible reason for the higher LOQ for morphine and 6-acetylmorphine is their extensive fragmentation in the collision cell, which causes dispersion of the ion current across several product ions (Figure 2). A detailed description of the complex fragmentation pattern of morphine and its derivatives can be found in the literature.[30,31] 11-nor-9-Carboxy-Δ9-THC had a relatively high IQL, but the low chemical noise in negative ion analysis resulted in a low LOQ.

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Table 4. Linear Ranges and Interday Correlation Factors of the Calibration Curves, Interday Instrumental Variability Measured on Two Different Standard Mixtures, and Intraday Instrumental Variability Measured in Wastewater[a]

illicit drug	linearity range, ng/μL	interday correlation factors (r^2) ± SD	interday RSD% 0.1 ng/μL	interday RSD% 1 ng/μL	intraday RSD% wastewater
benzoylecgonine	0.001−3	0.9998 ± 0.0002	5.14	0.97	3.50
norbenzoylecgonine	0.001−3	0.9996 ± 0.0005	7.78	0.78	6.56
cocaine	0.001−3	0.9998 ± 0.0002	4.18	0.91	1.79
norcocaine	0.001−3	0.9995 ± 0.0005	6.86	0.95	8.64
cocaethylene	0.001−3	0.9998 ± 0.0003	7.36	0.92	5.95
morphine	0.003−10	0.9998 ± 0.0003	9.81	1.35	2.84
6-acetylmorphine	0.001−10	0.9997 ± 0.0003	5.08	0.77	4.56
morphine−3β-D-glucuronide	0.001−3	0.9996 ± 0.0004	4.46	0.86	2.16
amphetamine	0.003−3	0.9996 ± 0.0003	9.34	0.60	2.88
methamphetamine	0.003−3	0.9997 ± 0.0002	9.46	0.06	2.51
MDA	0.003−3	0.9997 ± 0.0003	8.76	0.90	1.44
MDMA	0.003−3	0.9999 ± 0.0001	4.75	0.98	2.13
MDEA	0.003−3	0.9999 ± 0.0001	5.66	0.36	2.54
methadone	0.001−3	0.9999 ± 0.0001	3.98	1.00	2.24
EDDP	0.001−3	0.9999 ± 0.0001	5.39	1.06	3.47
11-nor−9-carboxy-Δ9-THC	0.001−3	0.9999 ± 0.0001	6.62	1.27	5.09

[a] Samples injected in triplicate.

The linearity of the analytical response was tested by using eight pooled standard solutions containing 0.001−10 ng/μL of each substance in Milli-Q water (0.01−100 ng of each substance injected; injection volume, 10 μL) (Table 4). Calibration curves for morphine and 6-acetylmorphine were linear in the whole range tested. The calibration curves for all other substances studied ceased to be linear when sample concentrations exceeded 3 ng/μL. Concentrations of illicit drugs and metabolites measured in wastewater were within this linearity range.

The repeatability of the calibration curves was determined by injecting pooled standard solutions three times during the same day and on different days. The interday correlation factors, r^2, of the calibration curves (means and SD) are reported in Table 4. The intraday values were >0.9992 with standard deviations <0.0005 (data not shown). The interday r^2 values were >0.9995 with SDs <0.0005.

Instrumental repeatability (interday RSD%) was assessed by triplicate injection of two standard mixtures with different concentrations of the substances (0.1 and 1.0 ng/μL) and of a wastewater sample (intraday RSD%). The results are reported in Table 4. The interday RSD% values were <1.3% for the 1.0 ng/μL concentration and <10% for 0.1 ng/μL. The intraday RSD% values were generally <5%, except for norbenzoylecgonine (6.5%), norcocaine (8.6%), and cocaethylene (5.9%).

Recoveries in wastewater were generally >80% (Table 3), with overall variability of the method, as indicated by the SD, <10% for the influents and <5% for the effluents.

Stability in Wastewater. Table 5 shows the results of the stability tests, expressed as percent differences between the initial and final concentrations of each illicit drug and metabolite after 3 days of storage at 4 °C. Cocaine, norcocaine, and cocaethylene concentrations decreased by 36, 15, and 13%, respectively, and their disappearence was paralleled by increases in the concentrations of their metabolites benzoylecgonine and norbenzoylecgonine (14 and 13%, respectively). This behavior was also observed in the control sample (no drugs added). Sample degradation was also observed for 6-acetylmorphine (14%) and morphine-3β-D-glucuronide (96%). The substantial decrease in morphine-3β-D-glucuronide was probably ascribable to its deconjugation by the β-glucuronidase enzymes of the fecal bacteria, as described for steroid conjugates.[32−34] In fact, the absolute decrease in morphine-3β-D-glucuronide (500 ng) corresponded to an equivalent increase in the morphine amount (taking into account the different spiking levels).

Table 5. Stability of the Most Abundant Drugs and Metabolites in Wastewater[a]

drug	amt spiked, ng/L	difference (%) after 3 days ± SD (%)
benzoylecgonine	5000	+13.9 ± 0.37
norbenzoylecgonine	500	+13.0 ± 0.27
cocaine	2000	−36.1 ± 2.19
norcocaine	500	−15.4 ± 0.82
cocaethylene	500	−13.0 ± 0.34
morphine	2000	+25.6 ± 1.45
6-acetylmorphine	500	−14.0 ± 0.53
morphine−3β-D-glucuronide	500	−96.3 ± 6.28
amphetamine	500	+4.9 ± 0.04
methamphetamine	500	+0.3 ± 0.01
MDA	500	−4.4 ± 0.15
MDMA	500	+0.8 ± 0.02
MDEA	500	−2.5 ± 0.05
methadone	500	+5.2 ± 0.11
EDDP	2000	+1.6 ± 0.01
11-nor−9-carboxy-Δ9-THC	2000	−7.8 ± 0.17

[a] Results are expressed as percent differences between initial and final concentrations of each substance after three days of storage at 4 °C.

Analysis of Wastewater. The analytical method was used to measure illicit drugs and their metabolites in WWTPs in Italy (Milan) and Switzerland (Lugano). Samples of untreated and treated wastewater (influents and effluents) were collected and

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Table 6. Concentrations (ng/L) of Illicit Drugs and Their Metabolites in Influents and Effluents of the Nosedo (Milan) and Lugano WWTPs

	Nosedo, February 2006		Lugano, March 2006	
illicit drugs	influent, mean[a] ± SD	effluent, mean[a] ± SD	influent, mean[a] ± SD	effluent, mean[a] ± SD
benzoylecgonine	1132.1 ± 197.2	<LOQ	547.4 ± 169.4	100.3 ± 28.6
norbenzoylecgonine	36.6 ± 7.8	<LOQ	18.8 ± 5.6	7.5 ± 2.9
cocaine	421.4 ± 83.3	<LOQ	218.4 ± 58.4	10.7 ± 3.2
norcocaine	13.7 ± 5.3	<LOQ	4.3 ± 0.9	0.7 ± 0.5
cocaethylene	11.5 ± 5.1	<LOQ	5.9 ± 2.6	0.2 ± 0.5
morphine	83.3 ± 11.8	<LOQ	204.4 ± 49.9	55.4 ± 11.1
6-acetylmorphine	11.8 ± 8. 5	<LOQ	10.4 ± 4.8	<LOQ
morphine−3β-D-glucuronide	2.5 ± 7.1	<LOQ	18.1 ± 30	<LOQ
amphetamine	14.7 ± 10.6	<LOQ	<LOQ	<LOQ
methamphetamine	16.2 ± 7.1	3.5± 2	<LOQ	<LOQ
MDA	4.6 ± 7.3	1.1 ± 1.5	<LOQ	0.9 ± 1.9
MDMA	14.2 ± 14.5	4.4 ± 3.7	13.6 ± 12.6	5.1 ± 3
MDEA	1.5 ± 3.8	<LOQ	<LOQ	<LOQ
methadone	11.6 ± 1.7	9.1 ± 0.5	49.7 ± 9.6	36.2 ± 2.8
EDDP	19.8 ± 3.1	22.6 ± 0.6	91.3 ± 19.2	72.1 ± 8.7
11-nor−9-carboxy-Δ9-THC	62.7 ± 5	<LOQ	91.2 ± 24.7	7.2 ± 3.7

[a] Mean of eight samples collected during 1 week.

analyzed as described. Results are shown in Table 6. In influents, benzoylecgonine and cocaine were the most abundant, with concentrations of about 1 μg/L and 0.5 μg/L for benzoylecgonine and 0.4 μg/L and 0.2 μg/L for cocaine, respectively, in Milan/Nosedo and in Lugano, whereas concentrations of the other metabolites of cocaine (norcocaine, norbenzoylecgonine, cocaethylene) were lower (4−36.6 ng/L). Morphine, which was found at relatively high concentrations (80−200 ng/L), may come from clinical use of morphine or codeine, but it might also come from illicit use of heroin. In fact, 6-acetylmorphine, a metabolite of heroin, was detected in influents from both plants (about 10 ng/L), suggesting widespread consumption of heroin.

Morphine is excreted in urine mainly as glucuronide metabolites, but morphine-3β-D-glucuronide was detected at low concentrations (2−18 ng/L), thus suggesting cleavage of the conjugated molecule in wastewater as previously discussed.[32,34] Concentrations of the amphetamines, methadone, and EDDP were generally lower than 20 ng/L, except in the Lugano plant, where methadone and EDDP reached 90 ng/L. Concentrations of 11-nor-9-carboxy-Δ9-THC in the influents were found to be 90 ng/L in the Lugano plant and ∼60 ng/L in the Milan plant.

Concentrations of drugs and metabolites were lower in effluents than in influents, particularly in the Milan plant, probably reflecting extensive degradation or sorption of these substances in WWTPs. However, significant amounts of illicit drugs and metabolites were still present in the Lugano effluents, and traces of the amphetamines and of methadone were also present in effluents from the Milan plant. Methamphetamine and MDMA concentrations in effluents were consistent with those previously detected in a U.S.A. study[28] (∼3−5 ng/L in our study and 0.5−2 ng/L in the U.S.A.), thus confirming illicit drugs and metabolites as widespread environmental contaminants.

CONCLUSION

An analytical method for the simultaneous determination of illicit drugs and their metabolites in wastewater was developed and applied to measure these substances in treated and untreated water from two WWTPs. The method allowed determinations in the low nanograms-per-liter range, with instrumental and overall variability <10%.

Cocaine and metabolites; amphetamines; morphine and metabolites; 11-nor-9-carboxy-Δ9-tetrahydrocannabinol, the main metabolite of tetrahydrocannabinol; and methadone and its main metabolite were found in substantial amounts in influents and effluents of both WWTPs. Illicit drugs can therefore be considered ubiquitous contaminants, discharged into the environment from WWTPs, as previously reported for pharmaceuticals by our group[35] and others,[36−37] with possible environmental risks.

Concentrations of these substances measured in WWTP influents may reflect consumption of illicit drugs by the local population, as we previously described for cocaine. The measurement of illicit drugs in WWTP influents might therefore offer a new tool for estimating drug abuse and consumption patterns in a community and monitoring trends and changing habits in real time while preserving the anonymity of the individuals involved.

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