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Direct injection ultra-performance liquid chromatography–tandem mass spectrometry for the high-throughput determination of etomidate and etomidate acid in wastewater

Shuai Yuan[a], Yue Xiao[a,b], Ruxin Luo[a], Xin Wang[a] and Ping Xiang[a,*]

[a] Department of Forensic Toxicology, Academy of Forensic Science, Shanghai Key Laboratory of Forensic Medicine, Shanghai Forensic Science Platform, Key Laboratory of Forensic Sciences, Ministry of Justice, Shanghai 200063, China
[b] School of Pharmacy, China Pharmaceutical University, Nanjing 211198, China
*Corresponding author. E-mail: xiangping2630@163.com

ABSTRACT

Etomidate (ET), a hypnotic agent used for the induction of anesthesia, is rapidly metabolized to etomidate acid (ETA) in the liver. Recently, ET has become one of the most serious alternative drugs of abuse in China. Therefore, an urgent need exists to develop a fast and convenient analysis method for monitoring ET. The current work presents a simple, fast, and sensitive direct injection method for the determination of ET and ETA in wastewater. After the optimization of the ultra-performance liquid chromatography–tandem mass spectrometry and sample filtration conditions, the method exhibited satisfactory limits of detection (1 ng/L) and good filtration loss. The validated method was successfully applied to determine the concentrations of ET and ETA in wastewater samples (n = 245) from several wastewater treatment plants in China. The concentrations of the targets in positive samples ranged from less than the lower limits of quantitation to 47.71 ng/L. The method can meet ET monitoring and high-throughput analysis requirements.

Key words: direct injection, etomidate, etomidate acid, UPLC–MS/MS, wastewater

HIGHLIGHT

- Etomidate (ET) use has spread rapidly, and it is now one of the most seriously abused substances in China. Our study first developed a simple, fast, and sensitive direct injection method for the quantitative determination of ET and etomidate acid in wastewater. After optimizing the UPLC–MS/MS and sample pretreatment conditions, the method was applied to authentic wastewater samples (n = 245) from several WWTPs in China.

1. INTRODUCTION

Etomidate (ET), a drug that induces sedation and sleep, was the first non-barbiturate intravenous anesthetic introduced on the market (Valk & Struys 2021). In the human body, ET is rapidly metabolized by esterases in the liver to form a hydrophilic phase 1 metabolite etomidate acid (ETA), which is then excreted by the kidneys (Molina *et al.* 2008). In China, ET has become one of a number of alternative substances of abuse due largely to the blocked entry, poor overall supply, and generally high prices of conventional mainstream drugs. ET use has spread rapidly in recent years, and it is now one of the most seriously abused substances in this country.

ET is usually added to electronic cigarette oil or shredded tobacco for smoking. Compared with traditional illicit drugs, ET has similar or stronger excitement, hallucinogenic, anesthesia, and other effects. Long-term use is associated with involuntary muscle activity, myoclonus, tremor, rigidity, chills, uncoordinated movement, and muscle tension (Valk & Struys 2021). Smoking large doses invokes mood changes, including bad temper and indolence, as well as altered thinking and behavior in those with mental disorders. Some users also develop manic symptoms and become prone to violent crimes. Long-term and large doses of ET can lead to confusion, coma, apnea, and death by suffocation. Given these dangers of its use, ET was listed as a regulated drug as of October 1, 2023 in China. Therefore, an urgent need exists to monitor the use of ET.

To our knowledge, research on ET and ETA has only been based on the analysis of blood, urine, hair, and other biological materials (Jung *et al.* 2019; Yum *et al.* 2021; Park *et al.* 2022). At present, no report has been published regarding a method for quantifying ET and/or ETA in wastewater, even though wastewater analysis can provide real-time, objective, and continuous

information about the use of drugs like ET. Wastewater analysis can also provide information about and comparisons of differences in ET consumption trends across time and space (Hall *et al.* 2012; Boogaerts *et al.* 2021). At present, wastewater analysis has played an important role in the monitoring of many illicit drugs, and it is currently undergoing comprehensive worldwide promotion (Yargeau *et al.* 2014; Tscharke *et al.* 2016; Goncalves *et al.* 2019; Brandeburová *et al.* 2020; Yuan *et al.* 2020; Liu *et al.* 2021).

In wastewater analysis, the majority of reported studies use solid-phase extraction (SPE) for sample pretreatment due to its high sensitivity and excellent capabilities for removing interfering impurities (Oestman *et al.* 2014; Foppe & Subedi 2018; Krizman-Matasic *et al.* 2018). However, SPE limits high-throughput analysis because the SPE protocol is laborious and time-consuming (Ng *et al.* 2020). Lately, direct injection methods have been applied to wastewater analysis as an effective method (Berset *et al.* 2010; Boix *et al.* 2015; Ren *et al.* 2022; Bade *et al.* 2023). In our laboratory, the direct injection method has been applied in routine illicit drug analysis (Ren *et al.* 2022). Compared to SPE, direct injection has several advantages, including simplified operation and reduced analysis costs for large quantities of actual samples. Therefore, we postulated that direct injection would be an effective method for the detection of ET and ETA in wastewater samples.

The aim of the current work was therefore to introduce and fully validate a simple, fast, and sensitive direct injection method for the determination of ET and ETA in wastewater. The method was successfully applied to samples collected from several wastewater treatment plants (WWTPs) in China.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Etomidate (ET), etomidate acid (ETA), and etomidate-D5 (ET-D5) were purchased from Cerilliant (Round Rock, TX, USA) as solutions in methanol (MeOH) at concentrations of 0.1 or 1 mg/mL. The isotopically labeled ET-D5 analog was used as an internal standard (IS). A mixed stock standard solution of ET and ETA (1 μg/mL) and the IS (100 ng/mL) were prepared by diluting with MeOH. Working standard solutions containing 10 and 0.2 ng/mL in water were prepared through serial dilutions of the stock standard solution. A working IS standard solution (10 ng/mL) was generated in water.

HPLC-grade MeOH was acquired from Supelco (Darmstadt, Germany). HPLC-grade acetonitrile and formic acid (98%) were acquired from ANPEL Scientific Instrument (Shanghai, China). Ammonium acetate was obtained from Fluka (Charlotte, NC, USA). Hydrochloric acid (analytical grade) was obtained from Yonghua Chemical Reagent Co., Ltd (Jiangsu, China). Ammonia solution (≥25% in water, analytical grade) was obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd (Shanghai, China). Ultrapure water was prepared using an in-house Milli-Q water system (Millipore, MA, USA). Mixed cellulose ester filters (MCE filters, 13 mm × 0.22 μm) and polytetrafluoroethylene filters (PTFE filters, 13 mm × 0.22 μm) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Polyethersulfone filters (PES filters, 13 mm × 0.45 μm) and Nylon filters (13 mm × 0.2 μm) were purchased from Shanghai ANPEL Scientific Instrument Co., Ltd (Shanghai, China).

2.2. Sample collection and treatment

The blank wastewater used for optimizing and validating the method was obtained from the inlet of a WWTP located in a small town in China. The blank sample was acidified with hydrochloric acid to pH 2, transported to the laboratory, and stored in the dark at −20 °C until analysis.

Authentic wastewater samples were collected every 2 h from various WWTPs using automatic sampling devices. Twelve wastewater samples collected on the same day were combined and stored in 600 mL PET bottles as a 24 h composite sample. In our study, all 31 WWTPs were sampled from September 29 to October 6, 2023 (a total of 245 wastewater samples). All wastewater samples were acidified with hydrochloric acid to pH 2, transported to the laboratory, and stored in the dark at −20 °C until analysis. Details of sampling information are included in Supplementary Table S1.

2.3. Sample pretreatment

We conducted a single-factor study to optimize multiple pretreatment conditions, including sample pH (pH 2, pH 4, pH 7, pH 9, and pH 11) and filter selection (PTFE, MCE, Nylon, and PES) to enhance the method's efficiency. The final sample pretreatment method was as follows: all samples were thawed to room temperature before pretreatment. A volume of 990 μL of wastewater sample was mixed with 10 μL of the IS working solution (10 ng/mL) and vortexed for about 30 s. The mixture

was then centrifuged at 13,400 rpm for 3 min. The supernatant was collected and filtered through a PTFE filter before injection. Each wastewater sample was analyzed in duplicate.

2.4. Ultra-performance liquid chromatography–tandem mass spectrometry conditions

Chromatographic separation was conducted using an Acquity™ UPLC I-CLASS PLUS system (Waters, USA). The targets were separated using a Restek Raptor Biphenyl (100 × 2.1 mm, 1.8 μm) column, with a SecurityGuard Raptor Biphenyl UHPLC (5 × 2.1 mm) column, at a flow rate of 0.3 mL/min. Mobile phase A consisted of 5 mmol/L ammonium acetate with formic acid (0.01%) in water, and mobile phase B was acetonitrile. A gradient elution program was applied as follows: 0–0.5 min, 5% B; 0.5–3 min, from 5 to 35% B; 3–6 min, from 35 to 95% B; 6–7 min, 95% B; 7–7.1 min, from 95 to 5% B; 7.1–8 min, 5% B. The total run time was 8 min. The injection volume was 20 μL.

Mass spectrometry analysis was performed using an AB SCIEX Triple Quadrupole™ 7500 Mass Spectrometer (AB SCIEX, USA) and run in a multiple reaction monitoring (MRM) mode and a positive ion mode. The MRM parameters are provided in Table 1. The ion source parameters were set as follows: curtain gas (CUR) was set at 40 psi, ion source temperature (TEM) at 400 °C, collision activation dissociation gas (CAD) at 5, ion spray voltage (ISV) at 1,400 V, ion source gas 1 (GAS 1) at 35 psi, and ion source gas 2 (GAS 2) at 60 psi. The data were evaluated using SCIEX OS.

2.5. Method validation

Method validation included selectivity, limits of detection (LODs), lower limits of quantitation (LLOQs), linearity, accuracy, precision, extraction recovery, and matrix effect, all evaluated under the optimized experimental conditions.

Selectivity was evaluated by analyzing six different blank wastewater samples. This evaluation was intended to demonstrate the potential interference of endogenous substances with the analyte or IS signals.

Linearity was achieved by analyzing eight calibration points in blank wastewater in the concentration range of 2–500 ng/L. The least square regression approach was applied to obtain a satisfactory correlation coefficient (r^2) > 0.99 based on the analyte/IS peak area ratio. The LODs were calculated by considering the signal-to-noise (S/N) ratio of at least 3:1 for the lowest concentration. The LLOQs were found from the S/N ratio of at least 10:1 at the lowest level in the calibration curve, and the LLOQs were required to have a relative standard deviation (RSD) of less than 20% and an accuracy ranging from 80 and 120%.

Precision and accuracy were evaluated using four different concentrations (2, 5, 50, and 400 ng/L) with six replicates at the calibration range. Accuracy was calculated based on the percentage ratio of the measured concentration to the nominal concentration. Intra-day precision and inter-day precision were determined by analyzing the RSD of spiked wastewater samples. Each concentration was evaluated over 4 days.

Recovery and matrix effect were assessed at three concentrations (5, 50, and 400 ng/L) with six replicates. The samples were divided into three groups: pretreatment spiked samples, post-treatment spiked samples, and neat solutions in the water. The recovery was calculated by dividing the pre-treatment spiked sample areas by the post-treatment spiked sample areas. The matrix effect was calculated by comparing the mean peak areas of the post-treatment spiked sample and the neat solutions in the water.

The stabilities of analytes were measured at two concentrations (5 and 400 ng/L) with six replicates under different storage conditions. The short-term stability was determined by exposing the samples to room temperature for 24 h. The samples stored at −20 °C for 3 weeks were used to assess the long-term stability. The post-preparative stability was evaluated after

Table 1 | Optimized MRM transitions and retention times for target compounds and IS

Number	Compound	Retention time (min)	Q1 (*m/z*)	Q3 (*m/z*)	Collision energy (V)
1	ET	5.22	245.1	**141.0**	14
				104.9	33
2	ETA	2.82	217.0	**113.0**	12
				94.9	33
	ET-D5	5.20	250.2	141.0	14

The quantifier ions are indicated in bold.

storage in an auto-sampler at 4 °C for 24 h. Finally, the freeze–thaw stability was tested after three freeze–thaw cycles (−20 to 25 °C).

3. RESULTS AND DISCUSSION

3.1. UPLC–MS/MS method optimization

Different gradients of mobile phase A and mobile phase B were used at a constant flow rate of 0.3 mL/min to ensure good peak shapes and separations. The LC optimization confirmed the suitability of an initial mobile phase consisting of mobile phase A: mobile phase B at 95%:5% (V/V) in a gradient pumped at 0.3 mL/min.

Four different MS parameters (TEM, CAD, GAS 1, and ISV) were optimized in this work to realize the optimum efficiency and sensitivity of this method (Fig. S1). When one parameter was being optimized, the other three remained unchanged. The MS optimization confirmed the great MS parameters: TEM at 400 °, CAD at 5, ISV at 1400 V, and GAS 1 at 35 psi.

3.2. Optimization of sample filtration

Five different sample pH values (pH 2, 4, 7, 9, and 11) were tested with six replicates in this work. Figure 1 shows the responses of the target compounds at the five different pH values. Overall, the responses of ETA had no significant change at all pHs, whereas the responses of ET were the best at pH 2. Thus, pH 2 was selected for sample pretreatment.

We optimized four different filters with six replicates for each target to improve the method's efficiency. As depicted in Figure 2, the losses of ET and ETA were lowest for filter D (PTFE filter, 13 mm × 0.22 μm; SCRC, China); therefore, filter D was used in this experiment.

3.3. Method validation

The selectivity of the method was evaluated using six different blank wastewater samples, which showed no responses attributed to the target compounds and IS.

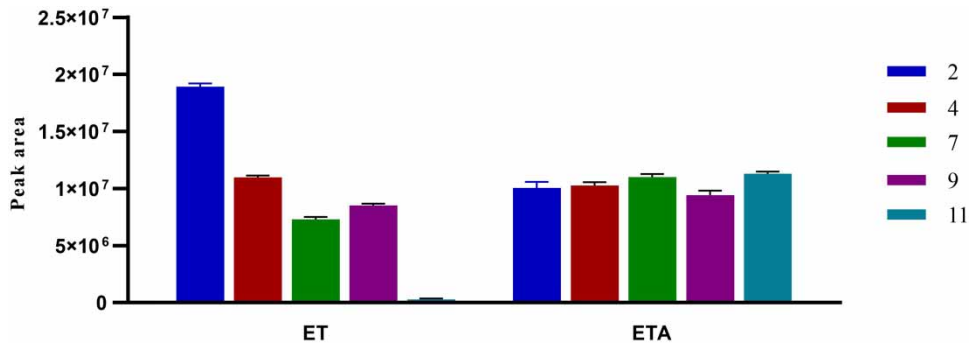

Figure 1 | Responses of target compounds pretreated at different pH conditions.

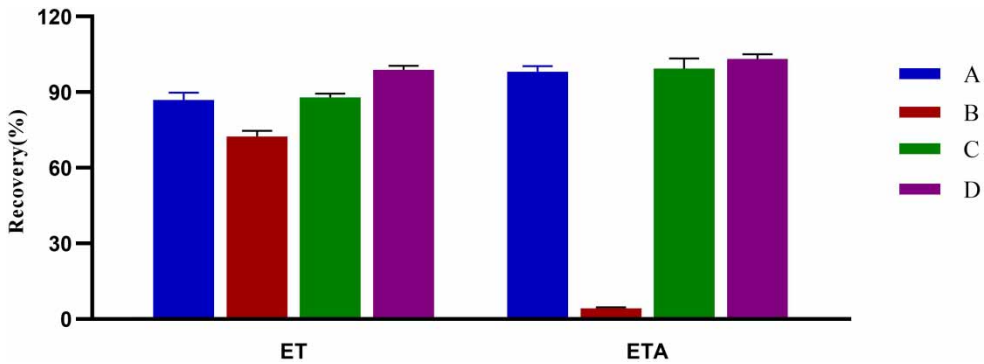

Figure 2 | Different filter recovery results for target compounds. A: PES filters (13 mm × 0.45 μm); B: Nylon filters (13 mm × 0.2 μm); C: MCE filters (13 mm × 0.22 μm; SCRC, China); D: PTFE filters (13 mm × 0.22 μm; SCRC, China).

In this study, the LODs were 1 ng/L and the LLOQs were 2 ng/L. The linear range was determined as 2–500 ng/L (Table 2). All correlation coefficients (r^2) exceeded 0.99. Figure 3 displays the chromatograms used for the LLOQs for ET and ETA in wastewater.

The results of the extraction recovery and matrix effect are shown in Table 3. The recoveries of ET and ETA ranged from 97.49 to 99.46% at 5 ng/L (low), 50 ng/L (medium), and 400 ng/L (high). The matrix effects for ET indicated signal enhancement (101.91–106.60%) and signal suppression (75.43–78.83%) for ETA.

As presented in Table 4, the intra-day precision was between 1.31 and 8.62%, and the accuracies varied between 95.10 and 108.73% ($n = 6$). The inter-day precisions ranged from 3.59 to 7.28% ($n = 24$). The inter-day and intra-day precision and accuracy values all met the acceptance criteria.

The stability results for all of the analytes investigated under different conditions (3 weeks at −20 °C; three freeze–thaw cycles; 24 h at 4 °C; 24 h at room temperature) are shown in Table S2. The mean concentration at each level was within 15% of the nominal concentration.

3.4. Application of the method to authentic wastewater samples

The validated method was successfully applied to determine the concentrations of ET and ETA in 245 wastewater samples from 31 WWTPs in China. The results for a total of 13 positive samples (3 WWTPs) are shown in Table 5. In these positive

Table 2 | Determination of the LODs, LLOQs, and linearity for targets in wastewater

Compound	Linearity range (ng/L)	Regression equations	Correlation coefficients (r^2)	LOD (ng/L)	LLOQ (ng/L)
ET	2–500	$y = 0.00488x + 0.00406$	0.99864	1	2
ETA	2–500	$y = 0.01100x + 0.00507$	0.99789	1	2

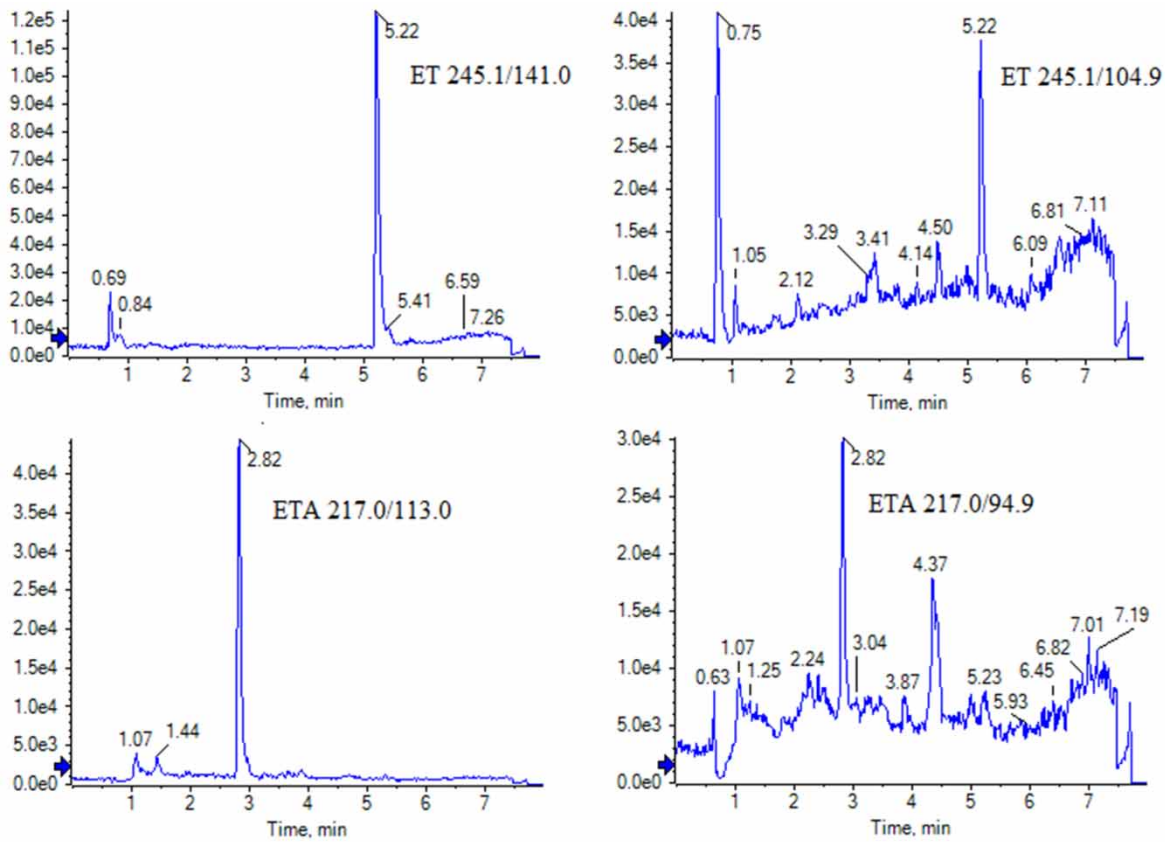

Figure 3 | Chromatograms of ET and ETA at LLOQ concentrations in wastewater.

Table 3 | Extraction recovery and matrix effect of targets in wastewater

Compound	Concentration (ng/L)	Etraction Recovery (%)	Matrix effect (%)
ET	5	99.46	102.17
	50	97.64	106.60
	400	97.49	101.91
ETA	5	98.94	75.43
	50	99.38	78.83
	400	98.92	77.68

Table 4 | Precision and accuracy of the targets in wastewater

Compound	Concentration (ng/L)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
ET	2	99.32	8.62	7.28
	5	102.41	1.31	3.92
	50	95.10	4.04	5.03
	400	98.14	4.31	3.59
ETA	2	104.39	4.42	5.13
	5	108.73	3.39	6.74
	50	104.17	3.77	5.99
	400	99.57	4.88	6.05

Table 5 | Concentrations of the targets in 13 wastewater samples (ng/L)

WWTP	Sampling date	ET	ETA
1	2023/10/1	3.99	2.65
	2023/10/2	3.09	2.47
	2023/10/3	2.74	5.03
	2023/10/4	3.34	3.39
2	2023/9/29	3.87	9.29
	2023/10/2	4.95	14
	2023/10/6	2.40	4.67
	2023/9/30	3.53	2.42
3	2023/10/2	27.78	4.22
	2023/10/3	5.27	7.76
	2023/10/4	5.00	24.97
	2023/10/5	35.14	47.41
	2023/10/6	3.29	<LLOQ

samples, ET was detected at concentrations ranging from 2.40 to 35.14 ng/L, while the concentrations of ETA ranged from <LLOQ to 47.71 ng/L. According to the information on WWTPs, WWTP 1 mainly treated medical wastewater from hospitals. ET, a drug that induces sedation and sleep, was a legal drug in the hospital. Therefore, the presence of ET and ETA in the inlet of WWTP 1 is reasonable. WWTP 2 and WWTP 3 mainly treated domestic wastewater from entertainment venues, including bars, karaoke TV, dance halls, etc. So, we may suspect that ET abuse may be occurring in some of the entertainment venues serviced by WWTP 2 and WWTP 3, and the details still need further investigation.

According to previous research, ET is metabolized to an inactive carboxylic acid metabolite (ETA) as a hydrophilic phase I metabolite (Van Hamme *et al.* 1978; Ghoneim & Van Hamme 1979; Fragen *et al.* 1983). The ETA is excreted in the urine and a small part in bile. Less than 2% of the ingested ET is excreted unchanged (Van Hamme *et al.* 1978). In our positive samples, the ratio of ET to ETA varied widely. This may be related to the fact that ET is added to e-cigarette oil or tobacco for smoking; therefore, a part of the ET does not enter the human body and may enter WWTPs directly. In addition, some positive sites may have legal or illegal ET production plants that may release ET wastes directly into the WWTPs. Alternatively, the large

ratio of ETA to ET may be related to the conversion of the targets in the sewers. We have carried out stability experiments, proving that ET and ETA were stable in wastewater for several days at low temperatures. However, no reports have been published regarding the conversion or biodegradation of ET and ETA in the sewer. Sewers are regarded as biological and chemical reactors, residence times of 30 min to 12 h (rarely up to 24 h) are typical in most catchments, and potential environmental processes may facilitate the formation of transformation products (McCall *et al.* 2016). Lin et al. (2021) evaluated the stability of 14 prescription drugs in sewers, and the result shows that the biodegradation of drugs in sewers with aerobic or anaerobic biofilms is higher than that in wastewater systems without biofilms. Consequently, some illicit drugs may be influenced by the prevailing environmental conditions in sewers. For example, Kinyua et al. (2018) investigated the in-sewer transformation products formed from synthetic cathinones and phenethylamines and found 18 transformation products. We will explore the conversion and biodegradation of ET and ETA in sewers in a future study.

Our findings confirm the usefulness of direct injection ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) for the simultaneous determination of ET and ETA in wastewater. The method presented here has the advantages of simple pretreatment, small injection volume, and high sensitivity to detect ET and ETA in wastewater. This is because the MS/MS sensitivity was improved with multiple novel hardware features. The OptiFlow Pro-Ion Source with E Lens Technology creates a stronger field at the Electrospray Ionization (ESI) probe, leading to a more efficient release of ions from the droplet and deflection of the ions toward the orifice. The D Jet Ion Guide behind the orifice plate also efficiently captures and transmits the ions in the higher vacuum region. Furthermore, the parameters for the target compounds, such as the mobile phase gradient, MS parameters, choice of filter membrane, and sample pH, were also optimized to achieve greater sensitivity. These features make our method exceptionally well-suited for analyzing extensive batches of wastewater samples.

No previous work has reported a method for the quantitative determination of both ET and ETA in wastewater. Table 6 summarizes the detection methods used to determine the content of other illicit drugs in wastewater. Overall, the direct injection method involves shorter pretreatment durations and demands a smaller sample volume when compared to conventional SPE approaches. The direct injection method clearly has great promise in wastewater analysis and could potentially play a pivotal role in illicit drug monitoring.

4. CONCLUSIONS

In this study, we developed a simple, fast, and sensitive direct injection method for the quantitative determination of ET and ETA in wastewater. After optimizing the UPLC–MS/MS and sample pretreatment conditions, the method was applied to

Table 6 | Summary of detection methods used for quantitative determinations of target illicit drugs in wastewater

Compound	Pretreatment method	Sample volume	Analytical operation time	LLOQ	Ref.
Seventeen synthetic cathinones	Offline SPE	50 mL	>60 min	0.11–1.57 ng/L	González-Mariño *et al.* (2016)
Eleven illicit drugs, including methamphetamine, morphine, and ketamine	Offline SPE	50 mL	>40 min	0.2–5 ng/L	Yuan *et al.* (2020)
Sixteen illicit drugs, including methadone, methamphetamine, and 3,4-methylenedioxymethamphetamine (MDMA).	Offline SPE	200 mL	>150 min	5–40 ng/L	Damien *et al.* (2014)
Twelve illicit drugs, including methamphetamine, cocaine, and codeine	Online SPE	2 mL	>18 min	0.5 ng/L	Wang *et al.* (2021)
37 psychoactive substances and illicit drugs, including lysergic acid diethylamide and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol	Online SPE	5 mL	>47 min	0.7–228 ng/L	López-García *et al.* (2018)
Eleven illicit drugs, including methamphetamine, morphine and ketamine	Direct injection	30 μL	11.5 min	1–5 ng/L	Ren *et al.* (2022)
ET and ETA	Direct injection	20 μL	11.5 min	2 ng/L	This work

authentic wastewater samples ($n = 245$) from several WWTPs in China. The concentrations of targets in the positive samples ranged from <LLOQ to 47.71 ng/L. The method can meet the requirements of ET monitoring and high-throughput analysis. Overall, direct injection UPLC–MS/MS has great potential as a significant tool for monitoring illicit drugs in the future.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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