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Rapid and simple LC–MS/MS determination of urinary ethyl glucuronide, naltrexone, 6β-naltrexol, chlordiazepoxide, and norchlordiazepoxide for monitoring alcohol abuse

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Abstract

In this study, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed to detect ethyl glucuronide (EtG), which is a biomarker for monitoring alcohol consumption, and naltrexone (NTX), 6β-naltrexol (6βNTX), chlordiazepoxide (CDP), and norchlordiazepoxide (norCDP), which are analyzed to confirm the presence of medications for alcohol dependence treatment. The protein precipitation method was conducted to rapidly prepare samples. LC–MS/MS analysis was performed in the multiple-reaction monitoring mode. The analytes were separated using a Scherzo SM-C18 (2.0 × 100 mm, 3 µm) column. The calibration ranges were 5–1000 ng/mL for EtG, 6βNTX, CDP, and norCDP, and 1–100 ng/mL for NTX, with the correlation coefficients (r) being ≥ 0.994, and the weighting factor being $1/x^2$. The lower limit of quantification was 1–5 ng/mL. The method was also validated for precision, accuracy, selectivity, dilution integrity, recovery, matrix effect, and stability. The developed method was successfully applied for the determination of EtG, NTX, 6βNTX, CDP, and norCDP in urine samples obtained from 49 probationers who received alcohol dependence treatment orders. The method developed herein can be used to monitor the drug-based treatment of alcohol abuse and alcohol consumption during the treatment of individuals under probation.

Keywords: Alcohol abuse, Ethyl glucuronide, Naltrexone, Chlordiazepoxide, Urine, LC–MS/MS

Introduction

Heavy or habitual drinking is one of the causes of physical diseases, mental disorders, and crimes. Crimes associated with alcohol intoxication, such as murder, sexual violence, arson, and traffic accidents, occur frequently, and uncontrolled drinking acts as a factor that increases the recidivism rate. In the Republic of Korea during 2020, 37.6% of 805 murders and 11.7% of 1155 incidents of sexual violence against children (< 13 years old) were committed by offenders under the influence of alcohol (Supreme Prosecutors' Office 2021). Treatment supervision and treatment order systems have been implemented in Korea to prevent the habitual alcohol consumption and recidivism of alcohol-intoxicated criminals. The treatment order system is a system in which the government intervenes and systematically manages drug treatment for the probationers to prevent repeated or more serious crimes (Kim et al. 2020; Korea Ministry of Justice 2020).

Ethyl glucuronide (EtG) is a direct metabolite of ethyl alcohol that can be detected in several matrices (urine, hair, blood, and meconium); it is one of the biomarkers used to monitor alcohol consumption (Biondi et al. 2019;

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Walsham and Sherwood 2012). EtG can be detected in the urine for 13–20 h with small alcohol intake (< 0.1 g/kg body weight). After heavy consumption of alcohol, it can be detected for up to 3–5 days (Ghosh et al. 2019, 2021). However, EtG can be detected in the urine following unintentional exposure to alcohol from commercial products (hand sanitizer, mouthwash, etc.), pharmaceuticals, and food (Gorgus et al. 2016; Rosano and Lin 2008). Therefore, clear criteria are required to avoid misidentification of unintentional alcohol exposure as alcohol consumption (Substance Abuse and Mental Health Services Administration 2012b; McDonell et al. 2015). The cutoff value of urinary EtG has been proposed to confirm alcohol consumption, which cannot be implemented for other alcohol biomarkers (Mercurio et al. 2021). The Substance Abuse and Mental Health Services Administration (SAMHSA) of the United States government has categorized the amount of urinary EtG detected as high positive (> 1,000 ng/mL), low positive (500–1,000 ng/mL), or very low positive (100–500 ng/mL) (Substance Abuse and Mental Health Services Administration 2012a). Meanwhile, the quantification of EtG in hair has established itself as a reliable biomarker of long-term alcohol consumption rather than that of ethyl sulfate (EtS) (Society of Hair Testing 2019). Published data suggest that EtG has potential as a highly sensitive and specific biomarker for the detection of alcohol abuse in both clinical and forensic chemistry.

Naltrexone (NTX), an opioid antagonist, is a drug used to treat patients suffering from alcohol dependence. NTX reduces the frequency of alcohol consumption and the relapse rate of individuals having alcohol use disorders by blocking the rewarding effect of alcohol consumption. Chlordiazepoxide (CDP) is a first-line benzodiazepine-class drug used to mitigate alcohol withdrawal symptoms. Prolonged use of CDP can lead to addiction and memory loss. Therefore, CDP is prescribed with a tapered dose (Liu et al. 2014; Nam Koong 2000; Ramanujam et al. 2015; Roozen et al. 2006). NTX and CDP are commonly used drugs to treat alcohol abuse according to the Korean alcohol guidelines for Primary Care Physicians (Jung et al. 2021).

Drug tests confirm the presence of drugs used to treat alcohol dependence through the detection and quantification of parent drugs and their metabolites in urine samples. The creatinine level should be measured because there is a possibility that the amount of EtG excreted through urine may be diluted and underestimated if the subject drinks a large amount of water (Wojcik and Hawthorne 2007). If the creatinine concentration is ≥ 2 mg/dL and < 20 mg/dL, and the specific gravity is > 1.001 and < 1.003, the urine is considered to be diluted (Chaturvedi et al. 2013). Sample pretreatment methods, such as solid-phase extraction (SPE), dilution, and precipitation, have been applied to minimize interference and selectively extract target analytes from urine. Analytical methods employing liquid chromatography–mass spectrometry (LC–MS), liquid chromatography–tandem mass spectrometry (LC–MS/MS), gas chromatography–mass spectrometry (GC–MS), and capillary electrophoresis have been developed and used for the determination of EtG and drugs used to treat alcohol dependence in biological samples. Recently, LC–MS/MS has become the most commonly used technique because it can analyze relatively polar analytes in biological samples without necessitating a derivatization process (Albermann et al. 2012; Bicker et al. 2006; Biondi et al. 2019; Cao et al. 2015; Concheiro et al. 2009; Ghosh et al. 2021; Morini et al. 2007; Politi et al. 2005; Saito et al. 2019; Vinkers et al. 2010).

In this study, a protein precipitation method was used to rapidly extract EtG, NTX, 6β-naltrexol (6βNTX), CDP, and norchlordiazepoxide (norCDP) from urine samples. The developed LC–MS/MS method was suitable for the simultaneous analysis of target compounds in urine samples. The method was used to evaluate the amount of alcohol consumed during alcohol-abuse drug treatment; it was applied to urine samples of 49 probationers who received the treatment order. The developed method will help reduce the incidence of alcohol-related crimes if it is used for periodic drug testing and facilitate the monitoring of alcohol consumption during the treatment for alcohol abuse.

Experimental

Chemicals and reagents

Ethyl glucuronide (EtG), naltrexone (NTX), 6β-naltrexol (6βNTX), chlordiazepoxide (CDP), norchlordiazepoxide (norCDP), and their deuterated internal standards (ISs), EtG-d_5, 6βNTX-d_3, CDP-d_5, and norCDP-d_5, respectively, were purchased from Cerilliant (Austin, TX, USA). The working solutions were prepared by sequentially diluting the standards according to the intended use and storing the diluted solutions at − 20 ℃ until use.

Methanol and water were purchased from J. T. Baker/Avantor (Center Valley, PA, USA), and formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Synthetic urine was purchased from CST Technologies, Inc. (UriSub®, Great Neck, NY, USA).

Urine samples

There is a limitation to validating analytical methods using human urine, because EtG can also be detected in urine following incidental ethanol exposure, including food, medicine, and skin exposure, instead of drinking (Gorgus et al. 2016; Rosano and Lin 2008). Therefore,

all validation parameters, except selectivity, were determined using a synthetic urine, 'UriSub®'. Synthetic urine has very similar physical properties to real urine, such as osmolality, specific gravity, and pH.

Urine samples of 49 probationers who received the medical treatment order requested by the probation office in Busan Metropolitan City and Gyeongsangnam-do Province from 2020 to June 2021 were used to determine alcohol abstinence during alcohol-abuse drug treatment. The samples were stored upon receipt at 4 ℃ and analyzed within 2 weeks.

Instruments

A Cobas C311 immunoassay analyzer (Roche, Hitachi) was used for creatinine analysis using the creatinine Jaffé gen.2 assay (CREJ2, Roche/Hitachi). A URISYS 2400 urine analyzer (Roche, Hitachi) was used to measure the specific gravity of the urine samples. An Agilent 1260 Infinity LC system (Palo Alto, CA, USA) equipped with an AB Sciex QTRAP 4500 MS system (Foster City, CA, USA) was used for LC–MS/MS analysis. A Scherzo SM-C18 (2.0 × 100 mm, 3 μm, Imtakt, Japan) column was used for chromatographic separation. Water containing 0.05% formic acid (mobile phase A) and methanol (mobile phase B) was used as the mobile phases, and the flow rate was 0.2 mL/min. The gradient conditions were as follows: Mobile phase B was maintained at 5% from 0 to 3 min, increased to 40% from 3 to 5 min, maintained at 40% until 7 min, and increased to 95% from 7 to 10 min. Finally, mobile phase B was decreased to 5% from 10 to 10.5 min and maintained at 5% until 20 min to stabilize the LC system. The column temperature was 25 ℃, and the autosampler temperature was 10 ℃.

Electrospray ionization (ESI) was used to generate ions for mass spectrometry. EtG and EtG-d5 were analyzed in the negative-ion mode, whereas the remaining analytes and ISs were analyzed in the positive-ion mode. The ion spray voltage was 5500 V, the ion source temperature was 550 ℃, and ion source gases 1 and 2 were supplied at 55 and 50 (arbitrary unit), respectively. The curtain gas was supplied at 30 (arbitrary unit), and the collision gas was used as the medium. The multiple-reaction monitoring (MRM) mode was used for the quantitative analysis. The retention time (RT), precursor ion, product ions, declustering potential (DP), entrance potential (EP), collision energy (CE), and collision-cell exit potential (CXP) of the standards and ISs were determined.

Sample preparation

Urine samples (100 μL) were mixed with methanol containing IS solutions (300 μL, a mixture of 400 ng/mL for EtG-d_5, 40 ng/mL for 6βNTX-d_3, 100 ng/mL for CDP-d_5, and 200 ng/mL for norCDP-d_5) and vortexed for 30 s. Following centrifugation at 30,000 g for 5 min, an aliquot (200 μL) of the supernatant was placed in a test tube for drying. The residue was redissolved in a mixture of methanol : water : formic acid (100 μL, 1:9:0.1 (v/v)), and an aliquot (5 μL) of the resulting sample was immediately injected into the LC–MS/MS instrument.

Validation of analytical method

The analytical method was validated for its selectivity, limit of detection (LOD), lower limit of quantification (LLOQ), linearity, accuracy, precision, dilution integrity, recovery (RE), matrix effect (ME), and stability according to the Bioanalytical Method Validation Guidance for Industry (Food and Drug Administration 2018).

Selectivity was confirmed by verifying whether interfering substances influence the retention times of the analytes and the ISs after analyzing 10 different urine samples.

The LOD and LLOQ were measured by determining the signal-to-noise (S/N) ratios using the standard deviations of the signals obtained from 10 urine samples to which a standard material was added, and the noises obtained from 10 blank samples. The LOD was defined as the lowest concentration at which the S/N ratio was 3 or higher, and the LLOQ was defined as the lowest concentration on the calibration curve having an accuracy of ± 20% and a precision of less than 20% while maintaining an S/N ratio of 10 or higher.

The calibration sample concentrations were 5, 15, 50, 100, 250, 500, 800, and 1000 ng/mL for EtG, 6βNTX, CDP, and norCDP, and 1, 3, 10, 20, 50, and 100 ng/mL for NTX. Linearity was confirmed via the correlation coefficient (r). A linear regression model incorporating a weighting factor of $1/x^2$ was applied to the calibration curves.

The repeatability of the analytical method was confirmed by measuring intra- and inter-day precision and accuracy. For precision and accuracy, quality control (QC) samples of four concentrations (LLOQ, low QC, medium QC, and high QC) were prepared and five samples of each concentration were measured in triplicate. LLOQs were within ± 20% for accuracy and 20% precision. The QCs of three concentrations were within ± 15% for accuracy and 15% precision.

The dilution integrity was evaluated by diluting QC samples corresponding to medium and high concentrations by 5, 10, 20, and 40 times. The diluted samples were divided into five aliquots and analyzed following the pretreatment.

The RE and ME were analyzed after preparing five samples each for sets A, B, and C. Set A was prepared by adding the analyte and the IS to the mobile phase, set B was prepared by adding the analyte and IS to the

eluent following the extraction of the blank sample, and set C was prepared by adding the analyte and IS to the blank sample and thereafter extracting it. The recovery (RE = C/B × 100) and matrix effect (ME = B/A × 100) were evaluated by calculating the ratios of the peak areas obtained by analyzing aliquots from each set.

The stability of analytes in urine samples was measured after preparing three samples, each corresponding to low QC and high QC. Bench-top stability was evaluated at room temperature for 24 h, and long-term stability was evaluated at 4 ℃ for 21 days. The autosampler stability was evaluated by re-injecting samples after storing vials containing the samples at 10 ℃ for 4 days.

Results and discussion

Sample preparation

In this study, protein precipitation was applied to rapidly prepare the samples for LC–MS/MS analysis. To compare removal of matrix in the urine samples, methanol and acetonitrile were used as organic solvents and the ratios of the urine to the organic solvent were set at 1:1, 1:3, and 1:5. When the ratio of urine:methanol was 1:3, the baseline of the chromatogram was low, and the intensity of the analyte peaks was the highest. Experiments for selecting solvents to redissolve the dried samples were conducted by changing ratios of water to methanol by increments of 10%. In the case of NTX, the intensity of the peak decreased as the methanol ratio increased and peak splitting occurred when the methanol ratio was 50% or higher (Fig. 1). When the methanol ratio was 10%, the shape and intensity of the peaks for all analytes were acceptable and the retention times were constant. In the case of EtG, the intensity was improved when formic acid was added. Upon changing the ratio of formic acid to 10% methanol in water from 0.05 to 0.5%, the highest peak intensity of EtG was observed at the ratio of 0.1%.

Optimization of LC–MS/MS conditions

The RT and shape of the peaks in the chromatogram were optimized by changing the column and mobile phases. The resolutions achieved using the following four types of columns were compared: Zorbax SB-C18 (2.1 × 75 mm, 3.5 µm), Scherzo SM-C18 (2.0 × 100 mm, 3 µm), Thermo Hypersil Gold (2.1 × 150 mm, 5.0 µm), and Waters Xselect HSS T3 (2.1 × 150 mm, 2.5 µm). Large differences in peak shape were observed for different column types in the case of EtG. Symmetrical peak shapes were obtained for EtG and other analytes when the Scherzo SM-C18 column was used (Fig. 2). Optimum mobile phase conditions were investigated using four different buffers (formic acid, acetic acid, ammonium formate, and ammonium acetate) and two different organic solvents (methanol and acetonitrile). The most symmetrical peak shapes of the analytes were obtained when formic acid was used as the buffer. Aqueous solutions with different formic acid concentrations in the range 0.01–0.1% were studied, and the peak retention time and intensity of the analytes were the highest at a formic acid concentration

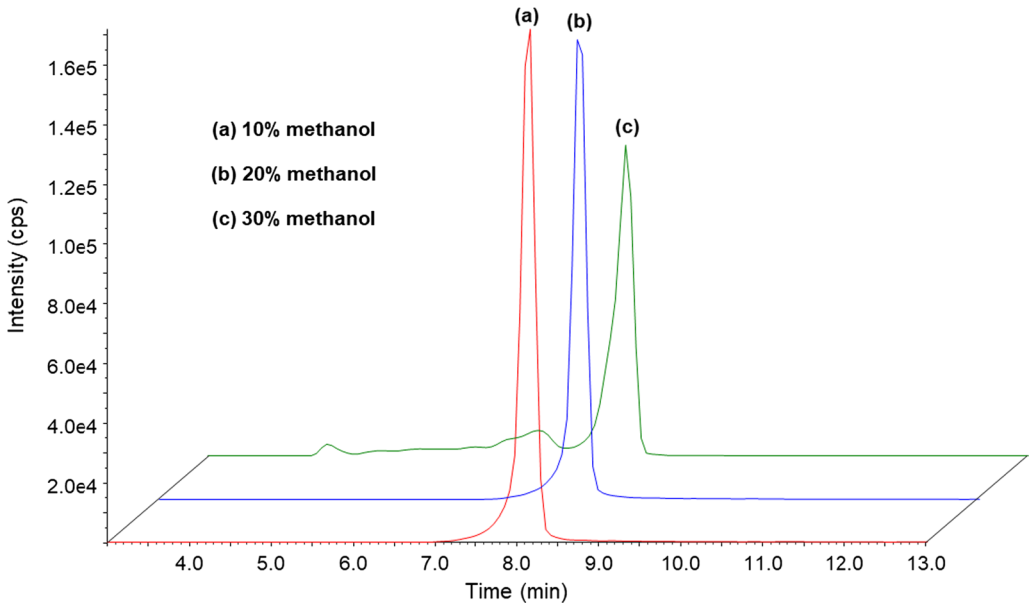

Fig. 1 Chromatograms of NTX at varying reconstituted solvent ratios used in the precipitation method **a** 10% methanol, **b** 30% methanol, and **c** 50% methanol

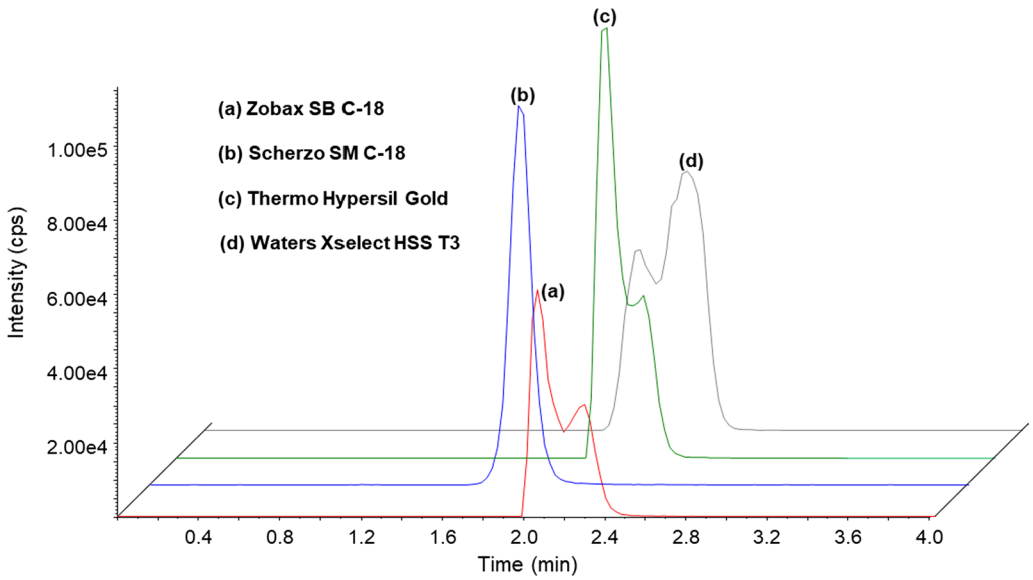

Fig. 2 Chromatograms of EtG for four types of columns: **a** Zorbax SB-C18 (2.1 × 75 mm, 3.5 µm), **b** Scherzo SM-C18 (2.0 × 100 mm, 3 µm), **c** Thermo Hypersil Gold (2.1 × 150 mm, 5.0 µm), and **d** Waters Xselect HSS T3 (2.1 × 150 mm, 2.5 µm). The columns were tested in isocratic mode (water containing 0.05% formic acid: methanol = 70:30) at flow rate of 0.2 mL/min

Table 1 Retention time (RT), MRM transitions, and mass spectrometric parameters

Compound	RT (min)	Precursor ion (*m/z*)	Product ion (m/z)	DP[a] (V)	EP[b] (V)	CE[c] (V)	CXP[d] (V)
EtG	5.14	221.0	75.0	− 55	− 10	− 18	− 7
			85.0	− 55	− 10	− 22	− 9
EtG-d_5	5.08	226.0	75.0	− 75	− 10	− 20	− 1
NTX	7.86	342.1	324.1	81	10	29	8
			270.0	81	10	39	10
6βNTX	8.08	344.1	308.0	76	10	39	10
			254.0	76	10	43	10
6βNTX-d_3	8.06	347.1	254.0	66	10	47	14
norCDP	11.30	286.0	269.0	86	10	21	12
			241.0	86	10	25	22
norDP-d_5	11.23	291.0	274.1	91	10	21	12
CDP	11.90	300.0	227.0	71	10	31	16
			283.0	71	10	33	14
CDP-d_5	11.85	305.0	232.0	81	10	31	6

[a] Declustering potential

[b] Entrance potential

[c] Collision energy

[d] Collision-cell exit potential

of 0.05%. For organic solvents, the peak intensity of the analytes was higher when using methanol than when using acetonitrile.

The analytes and ISs were analyzed using electrospray ionization (ESI). The MS/MS parameters were optimized to maximize the sensitivity toward the analytes by selecting their characteristic MRM ion pairs. Analytes that can be analyzed in the negative-ion mode (EtG and EtG-d_5) and those that can be analyzed in the positive-ion mode (NTX, 6βNTX, CDP, norCDP, 6βNTX-d_3, CDP-d_5, and norCDP-d_5) were concurrently analyzed by switching the ionization mode during sample analysis.

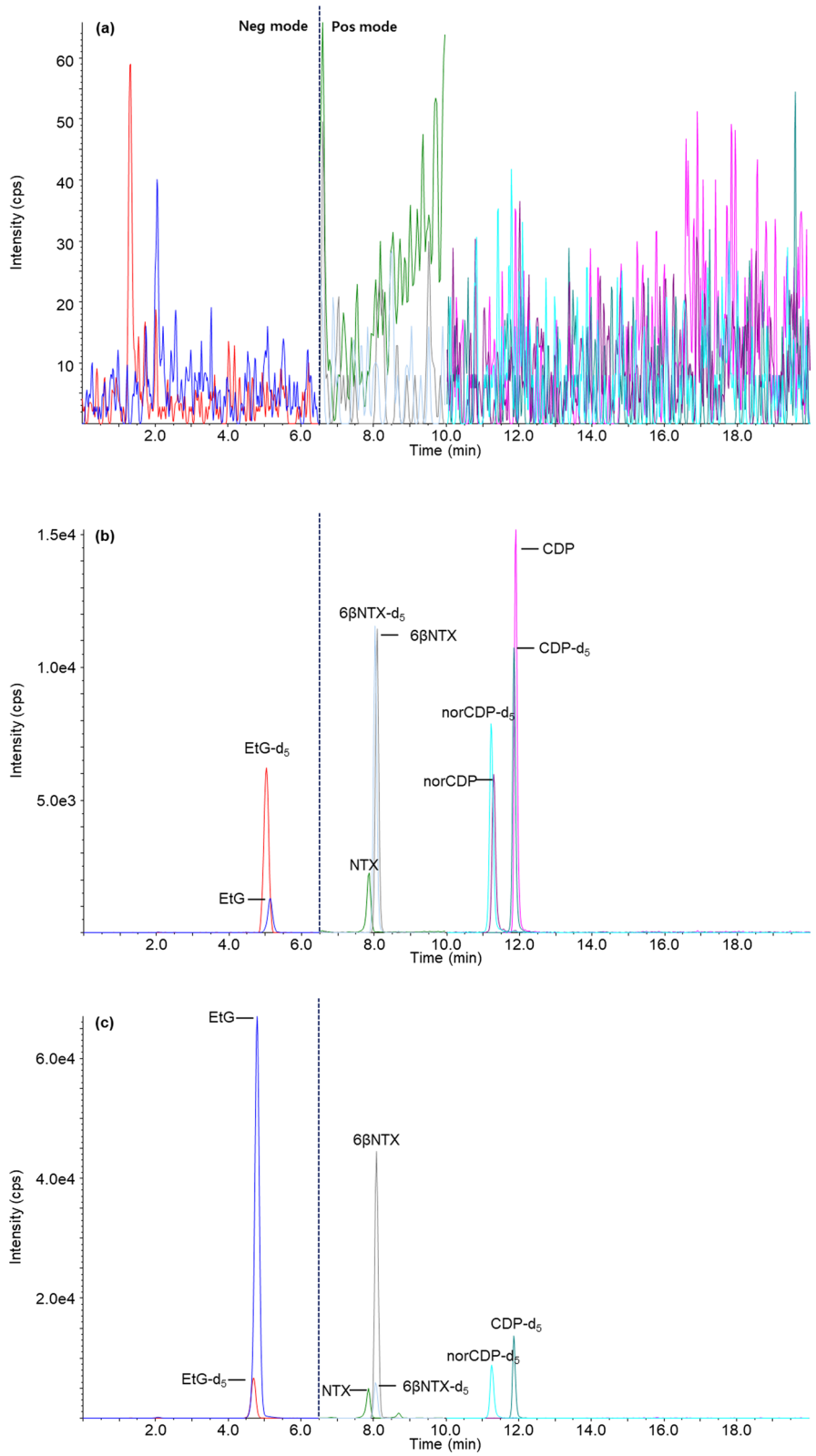

Fig. 3 Representative LC–MS/MS MRM chromatograms obtained from **a** blank urine, **b** spiked urine containing 50 ng/mL of EtG, 6βNTX, CDP, and norCDP, and 10 ng/mL of NTX, and **c** NTX-positive urine sample

Table 2 Limit of detection (LOD), lower limit of quantification (LLOQ), linearity, and calibration curve

Compound	LOD (ng/mL)	LLOQ (ng/mL)	Calibration range (ng/mL)	*r*	Slope	y-Intercept
EtG	1.5	5	5–1000	1.000	0.0016 ± 0.0001	0.0102 ± 0.0007
NTX	0.3	1	1–100	0.994	0.0854 ± 0.0083	− 0.0314 ± 0.0063
6βNTX	0.3	5	5–1000	0.994	0.0668 ± 0.0023	0.0117 ± 0.0707
CDP	1	5	5–1000	0.998	0.0209 ± 0.0007	− 0.0065 ± 0.0033
norCDP	1.5	5	5–1000	0.999	0.0103 ± 0.0005	− 0.0008 ± 0.0032

Table 3 Intra- and inter-day accuracy and precision, recovery (RE), and matrix effect (ME) used to validate the method using quality control (QC) samples

Compound	QC sample (ng/mL)	Intra-day ($n=5$)		Inter-day ($n=15$)		RE ($n=6$) %	ME ($n=6$) %
		% Bias	% CV	% Bias	% CV		
EtG	5	− 4.7	3.8	− 8.4	8.1	89.7	99.6
	15	− 1.4	6.7	− 0.2	7.1	90.6	102.0
	250	− 1.3	1.8	2.0	6.3	97.7	96.0
	800	− 0.3	0.9	3.4	6.8	98.0	98.3
NTX	1	7.5	6.5	7.8	6.8	95.9	52.2
	3	− 2.3	6.7	− 5.3	5.2	86.9	49.0
	20	− 2.2	6.3	− 3.8	6.8	103.0	50.3
	80	11.4	1.1	8.3	5.3	93.1	52.0
6βNTX	5	− 7.8	9.5	− 2.7	15.2	95.0	89.2
	15	− 7.3	7.2	− 1.0	10.1	88.6	88.1
	250	− 4.3	5.3	− 0.7	7.6	103.9	77.6
	800	0.1	4.3	− 4.4	6.7	104.2	71.7
CDP	5	11.2	5.2	2.3	10.0	89.7	48.9
	15	5.9	6.6	2.7	7.8	93.8	46.3
	250	− 0.4	7.0	1.3	5.9	96.6	47.0
	800	3.9	6.6	2.2	7.2	91.2	49.4
norCDP	5	− 5.0	5.3	− 0.5	6.3	95.3	45.3
	15	1.3	6.1	− 0.3	4.6	90.1	42.9
	250	8.8	3.0	2.8	5.6	103.0	42.1
	800	1.0	2.1	0.8	3.7	100.6	43.0

Samples were analyzed in the negative-ion mode from 0 to 6.5 min and analyzed up to 20 min after switching to the positive-ion mode. The mass spectrometric parameters for the analyses are listed in Table 1, and the chromatograms are shown in Fig. 3.

Validation of the method

The analytical method was validated by evaluating the selectivity, LOD, LLOQ, linearity, accuracy, precision, dilution integrity, RE, ME, and stability.

Based on the confirmatory results of the selectivity exhibited when analyzing urine samples ($n=10$) of the probationers who did not drink alcohol or take alcoholism-treatment medications, there were no interfering substances affecting the analysis of the analytes and ISs.

The calibration curve was linear within the range of quantification, with a correlation coefficient (r) of 0.994 or higher, and a weighting factor of $1/x^2$. The detection limit was 0.3–1.5 ng/mL, and the LLOQ was 1–5 ng/mL (Table 2).

The results of measuring the intra-day ($n=5$) and inter-day ($n=15$) precision and accuracy of the method were as follows: The LLOQ samples had an intra-day precision of 3.8–9.5%, intra-day accuracy of − 7.8 to 11.2%, inter-day precision of 6.3–15.2%, and inter-day accuracy

Table 4 Dilution integrity ($n = 5$)

Compound	Concentration before dilution (ng/mL)	Dilution factors	Concentration after dilution (ng/mL)	Determined concentration (ng/mL)	% Bias	% CV
EtG	250	10	25	24.4	− 2.5	8.2
		5	50	51.1	2.2	5.5
	800	40	20	20.6	2.8	6.8
		20	40	40.0	0.1	6.0
		10	80	80.6	0.7	2.0
NTX	20	10	2	2.0	− 1.4	4.9
		5	4	4.0	0.5	8.0
	80	40	2	1.9	− 5.4	3.2
		20	4	3.7	− 7.1	5.3
		10	8	7.3	− 8.3	5.2
6βNTX	250	10	25	25.2	0.9	8.3
		5	50	50.9	1.8	8.1
	800	40	20	19.0	− 5.2	7.9
		20	40	39.6	− 1.1	4.5
		10	80	76.8	− 4.0	7.3
CDP	250	10	25	24.1	− 3.6	9.4
		5	50	47.3	− 5.5	6.7
	800	40	20	19.6	− 2.2	4.7
		20	40	39.8	− 0.5	10.0
		10	80	81.8	2.2	4.8
norCDP	250	10	25	23.9	− 4.5	2.8
		5	50	50.2	0.4	3.4
	800	40	20	19.0	− 5.2	6.2
		20	40	38.6	− 3.4	8.7
		10	80	78.0	− 2.6	3.4

Table 5 Stability of the analytes ($n = 3$)

Compound	Concentration (ng/mL)	Autosampler stability (%) (10 ℃ for 4 days)	Bench-top stability (%)	Long-term stability (%)	
			(room temp. for 24 h)	(4 ℃ for 7 days)	(4℃ for 21 days)
EtG	15.0	− 4.0	0.3	0.7	− 2.7
	800.0	3.1	9.6	8.3	− 1.9
NTX	3.0	− 3.3	4.5	− 0.4	− 6.0
	80.0	0.1	2.0	0.7	− 2.1
6βNTX	15.0	− 4.3	− 7.3	− 4.9	− 4.0
	800.0	− 0.7	8.0	9.6	3.5
CDP	15.0	− 2.8	3.9	3.9	1.0
	800.0	2.5	0.4	4.3	− 5.8
norCDP	15.0	2.2	− 5.8	− 2.1	0.0
	800.0	3.4	− 0.1	5.0	− 4.5

of − 8.4 to 7.8%; the QC samples (LQC, MQC, and HQC) had an intra-day precision of 0.9–7.2%, intra-day accuracy of − 7.3 to 11.4%, inter-day precision of 3.7–10.1%, and inter-day accuracy of − 5.3 to 8.3%. The RE and ME are listed in Table 3.

The precision ($n = 5$) and accuracy ($n = 5$) measured by testing dilution integrity were 2.0–10.0% and − 8.3 to 2.8%, respectively (Table 4).

Table 5 shows the results of measuring the stability of analytes in the urine samples. The bench-top stability

Table 6 Quantification of EtG, NTX, and 6βNTX in urine samples from alcohol abusers ($n = 49$)

Item	EtG	NTX	6βNTX
Concentration (ng/mL)			
Range	6.3–34,654.7	1.5–3683.2	7.3–32,346.4
Median	227.6	183.4	8629.7
Mean	4649.8	511.4	9914.9
Normalized value[a] (ng/mg Cr)			
Range	11.2–46,033.0	1.1–3671.4	7.7–34,552.0
Median	183.0	154.0	8464.4
Mean	4310.8	510.1	9748.3
Frequency of taking drugs[b]	34		
Frequency of drinking[c]	8		

[a] Analyte concentration (ng/mL)/creatinine concentration (mg/dL) × 100

[b] Number of samples with concentrations exceeding the cutoff values. The cutoff values for NTX and 6βNTX were set to 10 ng/mL

[c] Number of samples with concentrations exceeding the EtG concentration of 1000 ng/mL. It was concluded that the subjects either drank heavily on the day of the test or on the previous day, or drank lightly on the day of the test

(room temperature for 24 h) was − 7.3 to 9.6%, and long-term stability (4 ℃ for 21 days) was − 6.0 to 3.5%. The stability of the liquid chromatography autosampler (10 ℃ for 4 days) was − 4.3 to 3.4%.

Application to forensic urine samples

Alcoholism treatment, drug use, and alcohol abstinence were confirmed using the urine samples of the probationers who received the medical treatment order. All 49 probationers were prescribed NTX. CDP was a first-line benzodiazepine-class drug used to mitigate alcohol withdrawal symptoms, but none of the probationers was prescribed CDP. Based on the creatinine level and specific gravity of all the urine samples, there was no diluted urine sample. The ratio of the creatinine to the analyte (analyte concentration (ng/mL) / creatinine concentration (mg/dL) × 100) was calculated to exclude inaccuracies caused by urine dilution for the analyte values measured in the urine samples (Fraser and Worth 2003). EtG was detected in 48 urine samples at concentrations of 6.3–34,654.7 ng/mL, and the normalized values were 11.2–46,033.0 ng/mg Cr. NTX and 6βNTX were detected in 38 urine samples at concentrations of 1.5–3683.2 ng/mL and 7.3–32,346.4 ng/mL, respectively, and the normalized values were 1.1–3671.4 ng/mg Cr and 7.7–34,552.0 ng/mg Cr, respectively. In previous studies, the cutoff values for NTX and 6βNTX were set to 10 ng/mL; if both the parent drug and its metabolite concentration of > 10 ng/mL were detected, it was considered that the drugs had been taken (Cao et al. 2015; Krock et al. 2017). As suggested by the SAMHSA, if an EtG concentration of > 1000 ng/mL (high positive) was detected, it was concluded that the probationers drank heavily on the day of the test or the previous day, or drank lightly on the day of the test (Substance Abuse and Mental Health Services Administration 2012a). Alcoholism-treatment drugs were not taken by 15 of the 49 probationers (30.6%). In addition, it was determined that eight of probationers (16.3%) who were under the treatment drank heavily on the day of the test or the previous day, or drank lightly on the day of the test. These results indicate that it is important not only to check whether the probationers who received the alcoholism treatment order are taking the treatment drugs regularly, but also whether they are drinking during the treatment (Table 6).

Conclusion

In this study, an analytical method for the rapid and simple determination of EtG, NTX, 6βNTX, CDP, and norCDP was developed to verify the medication compliance and the alcohol consumption by probationers who received the alcohol dependence treatment order. The protein precipitation method was conducted to rapidly prepare samples for LC–MS/MS. The proposed method was applied to the analysis of 49 urine samples obtained from probationers who received the medical treatment order. It was observed that 30.6% did not take their medication as directed and 16.3% drank alcohol. The developed method can be used to evaluate the amount of alcohol consumed during alcohol-abuse drug treatment.

Abbreviations

EtG: Ethyl glucuronide; EtS: Ethyl sulfate; NTX: Naltrexone; 6βNTX: 6β-Naltrexol; CDP: Chlordiazepoxide; norCDP: Norchlordiazepoxide; Cr: Creatinine; ISs: Internal standards; LC–MS/MS: Liquid chromatography–tandem mass spectrometry; LC–MS: Liquid chromatography–mass spectrometry; GC–MS: Gas chromatography–mass spectrometry; ESI: Electrospray ionization; MRM: Multiple-reaction monitoring; RT: Retention time; DP: Declustering potential; EP: Entrance potential; CE: Collision energy; CXP: Collision-cell exit potential; LOD: Limit of detection; LLOQ: Lower limit of quantification; QC: Quality control; LQC: Low quality control; MQC: Medium quality control; HQC: High quality control; RE: Recovery; ME: Matrix effect; S/N: Signal-to-noise; FDA: Food and Drug Administration; SAMHSA: Substance Abuse and Mental Health Services Administration.

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Authors' contributions

JY and YE contributed to the design of the study. YE performed the experiments and analyzed the data. JY, YE, JW, and BJ gave their valuable suggestions during the course of validation. YE wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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