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Rapid and quantitative screening method for 43 benzodiazepines and their metabolites, zolpidem and zopiclone in human plasma by liquid chromatography/mass spectrometry with a small particle column☆

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ABSTRACT

Benzodiazepines and their pharmacologically related drugs, zolpidem and zopiclone are widely prescribed as safe drugs, but these drugs are also abused in cases of crime, suicide and drug-facilitated sexual assault. We developed a rapid and quantitative screening method for 43 benzodiazepines, their metabolites, zolpidem and zopiclone in human plasma by liquid chromatography/mass spectrometry with a small particle column. All drugs were successfully separated within 12 min using combined scan and selected ion recording (SIR) mode. The calibration curves of most drugs were linear in the concentration range 0.5–250 ng/mL with correlation coefficients exceeding 0.99. Within-day precisions (RSD, %) of this method were 1.8–15.6% (10 ng/mL) and 0.6–10.1% (100 ng/mL) and between-day precisions (RSD, %) were 1.6–16.9% (10 ng/mL) and 0.6–16.7% (100 ng/mL). The average recoveries were 70.1% (10 ng/mL) and 87.1% (100 ng/mL). The limit of detection ranged from 0.2 to 8.0 ng/mL in 37 drugs and was below 0.2 ng/mL in 6 drugs. The established method is sensitive and rapid, thus it should be useful in forensic and clinical toxicological analyses.

1. Introduction

Benzodiazepines and their pharmacologically related drugs, zolpidem and zopiclone are widely prescribed as safe drugs with relatively few side effects for the treatment of insomnia, anxiety and epilepsy. The benzodiazepines bind to a non-specific site of the GABA receptor, leading to allosteric modification of this receptor. This binding amplifies the GABA affinity to the receptor site, resulting in an increased frequency of the chloride channel opening, thus hypo polarizing the membrane and bringing tranquilizing effect.

Benzodiazepines and their related drugs are also abused in cases of crime, suicide and drug-facilitated sexual assault. Lots of deaths and intoxications associated with these drugs occur in Japan [1,2], thus these drugs are often detected by the immunological screening kit, such as Triage® in either medico legal autopsy cases or emergency cases. However, subsequent qualitative and quantitative analyses using some instruments (generally, gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS)) are not always easy because of very low blood concentration and detection of multiple drugs at the same time.

Several analytical methods for the detection of multiple benzodiazepine drugs in human blood and urine samples have been reported using GC/MS [3–6], LC/MS [7–8] and LC/MS/MS [9–14]. Some of these drugs and their metabolites possess polar groups in a molecule which require derivatization prior to GC/MS analysis [3–5]. LC/MS(/MS) seems to be suitable for rapid simultaneous determination of these drugs comparing with GC/MS since extracted sample can be applied without derivatization and electro spray ionization (ESI) has capability to ionize most basic drugs [7,10–14]. However, long analytical time is often required in order to obtain sufficient chromatographic resolution of several tens of drugs using traditional LC/MS.

Recently, high through-put analysis named ultra performance liquid chromatography/mass spectrometry (UPLC®/MS) has been introduced [15–18]. High resolution, intensity and short analytical time can be obtained by using a high-pressure resistance column, which has narrower internal diameter and is packed with small particles under high-pressure analysis (up to 15,000 psi). Therefore, UPLC®/MS can be used for forensic and clinical analyses which require high sensitivity and analytical speed.

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We have developed a rapid and quantitative screening method for 43 benzodiazepines, their metabolites and benzodiazepine-related drugs in human plasma using this new analytical technique.

2. Experimental

2.1. Chemicals and reagents

7-Aminonitrazepam, 7-aminoclonazepam, 7-aminoflun itraze pam, N-desmethylflunitrazepam, 2-hydroxyethylflurazepam, desalkylflurazepam, 4-hydroxyalprazolam and diazepam-d_5 were purchased from Cerilliant (TX, USA). Midazolam, medazepam, flurazepam, clonazepam and alprazolam were purchased from Wako Pure Chemical Industries (Osaka, Japan). Zopiclone and delorazepam were purchased from Sigma–Aldrich Corp. (St Louis. MO, USA). Estazolam, N-desmethyldiazepam and diazepam were provided by Takeda Pharmaceutical Co., Ltd. (Osaka, Japan). Brotizolam, α-hydroxybrotizolam (WE964) and 4-hydroxybrotizolam (WE1061) were provided by Boehringer Ingelheim GmbH (Ingelheim, Germany). Triazolam, α-hydroxytriazolam and 4-hydroxytriazolam were provided by Pharmacia & Upjohn (MI, USA). Etizolam and clotiazepam were provided by Yoshitomi Pharmaceutical Co., Ltd. (Osaka, Japan). Nimetazepam and clobazam were provided by Dainippon Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan). Bromazepam, oxazepam and temazepam were provided by Hoffmann-La Roche Ltd. (Basel, Switzerland). Lormetazepam and lorazepam were purchased from Wyeth (NJ, USA). 3-Hydroxy-2-oxoquazepam (Quazepam-M4) and 2-oxoquazepam (quazepam-M5) were provided by Hisamitsu Pharmaceutical Co., Ltd. (Tokyo, Japan). Flunitrazepam was provided by Eisai Co., Ltd. (Tokyo, Japan). Zolpidem was provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Flumazenil was purchased from LKT Laboratories, Inc. (MN, USA). Fludiazepam was provided by Sumitomo Pharmaceuticals Co., Ltd. (Tokyo, Japan). Ethyl loflazepate was provided by Meiji Seika Ltd. (Tokyo, Japan). Chlordiazepoxide was provided by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). Cloxazolam and nitrazepam were provided by Sankyo Co., Ltd. (Tokyo, Japan). 8-Ethylhydroxyetizolam was provided by Mitsubishi Pharma Corporation (Osaka, Japan). Fig. 1 shows the structures of 43 drugs used in this study.

Acetonitrile (ACN) was purchased from Kanto chemical Co., Inc. (Tokyo, Japan). Formic acid and trifluoroacetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Focus™ column was purchased from Varian, Inc. (CA, USA).

2.2. Standard solutions

Most drugs (5 mg as free base) were dissolved in methanol, and volume was adjusted to 5 mL to obtain a concentration of 1000 μg/mL. This solution was further diluted in methanol to 100, 10, and 1 μg/mL. 7-Aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, desalkylflurazepam, N-desmethylflunitrazepam, 2-hydroxyethylflurazepam and diazepam-d_5 (1000 μg/mL ampule) were further diluted in methanol to 100, 10, and 1 μg/mL. 8-Ethylhydroxyetizolam, 4-hydroxyalprazolam, α-hydroxytriazolam and 4-hydroxytriazolam (100 μg/mL ampule) were further diluted in methanol to 10, and 1 μg/mL. Zopiclone was dissolved in ethanol because of instability in methanol [8].

2.3. Biological samples

Plasma samples were obtained from healthy Japanese volunteers and stored at −20 °C until analysis.

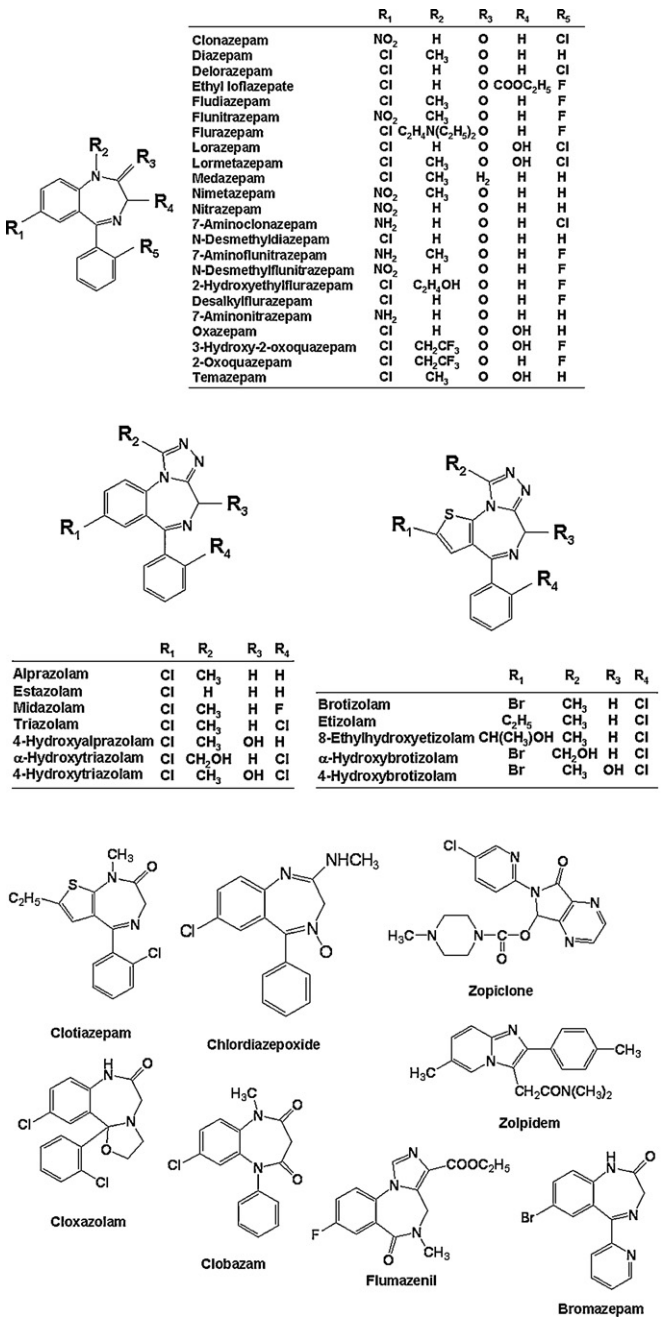

Fig. 1. Structures of 43 drugs used in this study.

2.4. Sample preparation

One milliliter of plasma was mixed with 10 μL IS solution containing 10 μg/mL of diazepam-d_5 in a centrifuge tube, and 300 μL of 1 M acetic acid buffer (pH 5.0) and 1 mL of distilled water were added. The mixture was vortex-mixed for 30 s, sonicated for 10 min and centrifuged at 850 × *g* for 5 min. The supernatant was applied to a Focus™ column previously conditioned sequentially with 1 mL of methanol and 1 mL of distilled water. The column was rinsed sequentially with 1 mL of distilled water and 1 mL of 10% acetonitrile. The analytes were eluted sequentially with 0.75 mL of 0.1% trifluoroacetic acid in ACN solution and 0.75 mL of 0.2% ammonia in ACN solution. The eluate was evaporated to dryness under a stream of nitrogen at 60 °C. The residue was dissolved in 100 μL of initial mobile phase, filtered through Acrodisc GHP (0.2 μm pore size and 13 mm diameter, Waters)

and a 5 μL aliquot of the solution was subjected to analysis by LC/MS.

2.5. LC conditions

The apparatus used was an Acquity UPLC system (Waters, MA, USA). The gradient elution was performed on Van guard pre-column (Waters, 5 mm × 2.1 mm, 1.7 μm) and an Acquity UPLC BEH C18 column (Waters, 100 mm × 2.1 mm, i.d., 1.7 μm). The mobile phase consisted of two solvents; solvent A was 0.05% formic acid aqueous solution, and B was 0.05% formic acid in ACN. The gradient was programmed as follows: 0–0.5 min 25% B, 1.0–6.0 min 30% B, 9.5 min 40.5% B, 11.0–13.0 min 90% B, 13.01–17 min 25% B. The flow-rate was 0.4 mL/min.

2.6. MS conditions

The apparatus was a ZQ 2000 mass detector with an ESI in the positive ionization mode. The ESI inlet conditions were as follows: ion source temperature, 120 °C; capillary voltage, 3.5 kV; desolvation temperature, 350 °C; desolvation gas flow, 50 L/h; cone gas flow, 50 L/h. The scan mode measurement combined with selected ion recording (SIR) mode was performed. Table 1 shows the category, retention time (RT), $[M+H]^+$, mode of measurement and cone voltage of each drug. The cone voltage was 45 V and scan range was from m/z 200 to 500 at scan time of 0.4 s in scan mode. $[M+H]^+$ ion was used as monitoring ion for each drug. The cone voltages and monitoring ions in SIR mode were 25 V and m/z 389 for zopiclone, 45 V and m/z 308 for zolpidem, 30 V and m/z 388 for flurazepam, 45 V and m/z 316 for clonazepam, 50 V and m/z 343 for triazolam, 45 V and m/z 395 for brotizolam and 30 V and m/z 335 for lormetazepam. The occurrence of each drug was screened by detecting the peak of monitoring ion $[M+H]^+$ at around the retention time (within ±0.2 min).

2.7. Validation

Plasma samples were prepared to contain: 24 drugs at concentrations of 0.5, 1.0, 5.0, 10, 50, 100 and 250 ng/mL; 8 drugs at concentrations of 1.0, 5.0, 10, 50, 100 and 250 ng/mL; 1 drug at concentrations of 5.0, 10, 50, 100, 250 ng/mL; 8 drugs at concentrations 10, 50, 100, 250 ng/mL each containing 100 ng of IS. These samples were extracted in the same manner as described above. The calibration curves were obtained by plotting the peak-area ratio of the specific drug to IS versus the amount of that drug (Table 2).

The absolute recoveries of each drug in plasma samples at two different concentrations, 10 and 100 ng/mL, were determined by comparing the average peak area of each drug in samples ($n = 8$) with that in standard solution ($n = 2$). QC samples were prepared

Table 1
Name, category, RT, $[M+H]^+$, measurement mode and cone voltage of 43 drugs.

Drug name	Category	RT (min)	$[M+H]^+$	Mode	Cone voltage (V)
7-Aminonitrazepam	Metabolite	0.76	252	SCAN	45
Zopiclone	Hypnotic	1.04	389	SIR	25
7-Aminoclonazepam	Metabolite	1.14	286	SCAN	45
Cloxazolam	Antianxiety	1.15	349	SCAN	45
7-Aminoflunitrazepam	Metabolite	1.41	284	SCAN	45
Zolpidem	Hypnotic	1.56	308	SIR	45
Chlordiazepoxide	Antianxiety	1.92	300	SCAN	45
8-Ethylhydroxyetizolam	Metabolite	2.18	359	SCAN	45
Flumazenil	Benzodiazepines antagonist	2.26	304	SCAN	45
Bromazepam	Antianxiety	2.76	316	SCAN	45
Midazolam	Hypnotic	3.00	326	SCAN	45
Medazepam	Antianxiety	3.09	271	SCAN	45
Flurazepam	Hypnotic	3.45	388	SIR	30
4-Hydroxyalprazolam	Metabolite	4.63	325	SCAN	45
N-desmethylflunitrazepam	Metabolite	4.80	300	SCAN	45
Nitrazepam	Hypnotic	4.85	282	SCAN	45
α-Hydroxytriazolam	Metabolite	5.01	359	SCAN	45
α-Hydroxybrotizolam	Metabolite	5.21	411	SCAN	45
4-Hydroxytriazolam	Metabolite	5.23	359	SCAN	45
Oxazepam	Metabolite	5.41	287	SCAN	45
Estazolam	Hypnotic	5.50	295	SCAN	45
4-Hydroxybrotizolam	Metabolite	5.68	411	SCAN	45
Clonazepam	Antiepileptic	5.86	316	SIR	45
Lorazepam	Antianxiety	6.08	321	SCAN	45
2-Hydroxyethylflurazepam	Metabolite	6.22	333	SCAN	45
N-desmethyldiazepam	Metabolite	6.33	271	SCAN	45
Alprazolam	Antianxiety	6.53	309	SCAN	45
Flunitrazepam	Hypnotic	7.03	314	SCAN	45
Nimetazepam	Hypnotic	7.07	296	SCAN	45
Triazolam	hypnotic	7.18	343	SIR	50
Desalkylflurazepam	Metabolite	7.23	289	SCAN	45
Temazepam	Metabolite	7.87	301	SCAN	45
Brotizolam	Hypnotic	7.96	395	SIR	45
Etizolam	Antianxiety	8.13	343	SCAN	45
Clobazam	Antiepileptic	8.41	301	SCAN	45
Delorazepam	Metabolite	8.51	305	SCAN	45
Lormetazepam	Hypnotic	8.79	335	SIR	30
Clotiazepam	Antianxiety	9.03	319	SCAN	45
Diazepam-d_5	IS	9.15	290	SCAN	45
Diazepam	Antianxiety, antiepileptic	9.29	285	SCAN	45
Fludiazepam	Antianxiety	9.51	303	SCAN	45
3-Hydroxy-2-oxoquazepam	Metabolite	10.55	387	SCAN	45
Ethyl lofrazepate	Antianxiety	10.67	361	SCAN	45
2-Oxoquazepam	Metabolite	11.09	371	SCAN	45

Table 2
Peak number, calibration range, correlation coefficient, within-day and between-day precision, recovery and limit of detection of 43 drugs in this method.

Peak no.	Drug name	Calibration range (ng/mL)	r^2	10 ng/mL				100 ng/mL				Limit of detection (ng/mL)
				Precision (RSD (%), $n=5$)		Recovery ($n=8$, %)		Precision (RSD (%), $n=5$)		Recovery ($n=8$, %)		
				Within-day	Between-day	Average (range)	SD	Within-day	Between-day	Average (range)	SD	
1	7-Aminonitrazepam	10–250	0.999	9.8	3.5	15.1 (11.8–18.3)	2.4	5.5	14.1	31.1 (28.2–33.8)	2.0	3
2	Zopiclone	0.5–250	0.997	5.6	14.0	72.6 (66.2–78.6)	5.5	1.6	10.0	85.5 (80.9–90.4)	3.2	0.4
3	7-Aminoclonazepam	10–250	0.995	5.1	8.7	39.4 (30.2–46.6)	5.0	8.1	10.4	65.7 (67.7–73.4)	4.1	2
4	Cloxazolam	1.0–250	0.997	8.9	10.0	70.3 (65.4–76.5)	3.5	3.8	7.6	82.4 (77.8–89.1)	3.4	1
5	7-Aminoflunitrazepam	0.5–250	0.999	9.7	8.0	41.4 (35.7–50.8)	5.1	2.2	11.1	70.4 (65.4–76.9)	3.3	0.4
6	Zolpidem	0.5–250	0.999	15.6	6.9	90.8 (77.9–99.5)	7.0	4.8	3.6	99.5 (97.0–105.8)	3.0	<0.2
7	Chlordiazepoxide	10–250	0.996	4.1	7.5	58.8 (43.1–78.2)	15.1	3.9	9.0	96.6 (79.9–110.4)	8.9	4
8	8-Ethylhydroxyetizolam	0.5–250	0.998	5.8	7.4	72.4 (64.1–77.5)	4.5	3.0	9.5	83.7 (78.0–91.2)	4.2	0.5
9	Flumazenil	1.0–250	0.993	7.0	2.4	75.8 (72.4–82.1)	4.5	2.3	11.1	99.7 (94.4–103.7)	5.9	<0.2
10	Bromazepam	10–250	0.996	5.0	15.7	52.7 (40.2–69.3)	9.3	5.6	14.6	76.5 (70.7–88.9)	5.9	8
11	Midazolam	0.5–250	0.998	8.3	6.4	67.0 (58.8–69.9)	6.6	0.6	8.3	88.6 (82.9–93.1)	3.3	0.5
12	Medazepam	1.0–250	0.999	4.0	3.2	54.7 (35.1–82.2)	17.4	6.9	3.0	80.0 (61.2–91.7)	10.8	0.5
13	Flurazepam	0.5–250	1.000	8.7	9.8	91.3 (84.0–96.6)	4.5	3.9	2.2	103.4 (95.1–108.0)	3.8	0.2
14	4-Hydroxyalprazolam	1.0–250	0.996	5.7	13.2	63.3 (50.3–76.6)	7.6	7.5	16.7	65.8 (59.0–77.1)	6.0	1
15	N-desmethylflunitrazepam	0.5–250	1.000	5.6	12.1	74.9 (63.8–82.3)	5.7	2.2	8.8	89.2 (80.5–96.1)	4.6	0.5
16	Nitrazepam	0.5–250	0.999	6.8	7.9	84.1 (71.7–97.1)	9.3	3.4	9.7	98.3 (88.0–103.4)	4.8	0.5
17	α-Hydroxytriazolam	1.0–250	1.000	12.1	16.9	74.2 (63.1–88.4)	7.4	4.9	13.2	94.3 (93.9–100.7)	5.1	1
18	α-Hydroxybrotizolam	1.0–250	0.998	9.1	7.7	83.8 (76.9–95.1)	7.3	5.0	6.1	101.3 (95.6–108.6)	4.5	1
19	4-Hydroxytriazolam	1.0–250	0.996	8.6	9.8	81.8 (76.3–92.3)	5.4	2.4	6.2	93.0 (84.4–100.6)	4.8	1
20	Oxazepam	10–250	1.000	3.3	6.7	57.0 (46.7–67.9)	8.5	2.5	4.9	80.2 (67.1–85.3)	7.0	4
21	Estazolam	0.5–250	0.999	6.9	14.4	58.3 (52.2–70.7)	8.1	2.3	9.1	79.1 (67.9–85.5)	5.7	0.5
22	4-Hydroxybrotizolam	10–250	0.996	13.6	4.6	72.4 (59.2–86.7)	11.6	3.8	8.0	96.8 (83.9–102.5)	6.9	4
23	Clonazepam	0.5–250	0.997	4.7	3.6	90.5 (82.9–97.7)	5.5	6.4	1.6	103.7 (96.7–108.3)	5.3	<0.2
24	Lorazepam	0.5–250	0.998	6.9	4.2	81.8 (69.6–93.8)	9.0	7.0	2.1	98.4 (88.4–102.9)	4.8	<0.2
25	2-Hydroxyethylflurazepam	0.5–250	0.997	5.2	8.6	87.4 (67.6–158.7)	29.1	2.7	10.0	94.2 (88.9–98.6)	3.4	0.5
26	N-desmethyldiazepam	1.0–250	1.000	2.7	4.4	65.7 (47.7–85.7)	14.4	6.3	5.9	90.4 (72.5–100.7)	9.5	0.8
27	Alprazolam	0.5–250	1.000	7.5	4.2	55.9 (48.0–70.5)	9.1	10.1	9.1	78.7 (71.1–85.2)	4.9	0.2
28	Flunitrazepam	10–250	0.999	5.4	1.6	85.5 (74.6–93.2)	7.0	2.7	7.3	101.5 (95.5–104.3)	2.8	2
29	Nimetazepam	1.0–250	0.999	4.1	11.8	87.7 (78.6–95.0)	5.8	0.7	11.0	103.2 (99.7–108.5)	3.0	0.8
30	Triazolam	0.5–250	0.998	3.3	8.3	81.9 (72.4–87.2)	5.2	5.2	3.9	98.2 (93.1–102.6)	3.9	<0.2
31	Desalkylflurazepam	0.5–250	0.999	4.7	7.1	58.0 (46.9–76.8)	12.8	4.8	4.9	82.6 (77.5–89.5)	7.5	<0.2
32	Temazepam	0.5–250	1.000	4.6	4.9	42.6 (32.1–56.4)	9.5	2.1	2.0	66.7 (56.1–79.5)	6.7	0.2
33	Brotizolam	0.5–250	0.997	6.2	8.1	78.0 (65.7–89.3)	8.8	5.9	2.5	99.4 (94.7–104.1)	4.0	0.2
34	Etizolam	0.5–250	0.998	6.8	5.9	80.7 (68.6–94.8)	10.5	2.1	7.3	99.9 (95.7–103.5)	2.8	0.5
35	Clobazam	0.5–250	0.999	7.1	6.0	82.6 (71.3–95.9)	9.8	3.5	7.1	102.5 (89.6–110.4)	7.3	0.4
36	Delorazepam	0.5–250	0.999	1.8	15.4	72.4 (59.0–85.8)	10.3	3.1	8.5	99.9 (89.3–106.7)	5.8	0.5
37	Lormetazepam	0.5–250	0.998	6.7	5.4	67.8 (53.1–84.2)	12.5	8.9	4.0	97.5 (78.9–109.7)	10.4	0.4
38	Clotiazepam	0.5–250	0.996	2.9	5.8	34.3 (18.5–51.3)	12.2	1.3	1.0	56.8 (40.8–65.8)	9.0	0.5
39	Diazepam	1.0–250	0.997	4.3	5.2	39.1 (22.6–59.0)	13.7	1.8	0.6	65.0 (56.5–76.7)	9.5	0.6
40	Fludiazepam	0.5–250	0.995	2.5	3.2	39.0 (23.3–56.8)	12.8	2.5	2.9	63.4 (46.6–69.7)	8.6	0.5
41	3-Hydroxy-2-oxoquazepam	10–250	0.998	4.4	12.5	102.9 (91.1–107.9)	11.0	4.2	7.5	95.1 (83.0–98.6)	5.0	1
42	Ethyl lofrazepate	0.5–250	0.998	7.2	8.2	124.9 (73.2–158.9)	31.6	5.2	6.2	92.5 (77.0–104.4)	9.4	0.5
43	2-Oxoquazepam	5–250	0.998	6.8	6.2	103.2 (83.8–125.3)	13.3	7.3	1.4	95.2 (83.3–101.7)	5.9	4

at concentrations of 10 and 100 ng/mL and analyzed as described above. Within-day and between-day precisions ($n = 5$, as relative standard deviation, RSD (%)) were calculated based on the prepared calibration curves. The limit of detection (LOD) for each drug was estimated at a signal-to-noise ratio equal to three in spiked plasma.

The matrix effect ($n = 2$) on the ESI response was evaluated by analyzing two different samples: 10 and 100 ng of each drug (a) and the same amount of drug added to pre-extracted three different sources of plasma samples (b). Both samples were dissolved in initial mobile phase and submitted to LC/MS as described above. The peak area obtained from sample (a) provides a relative 100% value and ion suppression (%) was defined as $100 \times (a - b)/a$.

3. Results and discussion

Solid phase extraction has some merits for sample preparation compared with liquid–liquid extraction: (1) very simple extraction procedure, (2) necessity of low amount of solvent volume, (3) high through-put performance and feasibility for treatment of a lot of samples at one time, (4) minimization of differences among individuals. We have confirmed the usefulness of the Focus™ column for extraction of abused drugs from biological fluids in the past studies [19–21]. This column holds compounds with four interactions, hydrogen bond interaction (donor and acceptor), dipole–dipole interaction and hydrophobic interaction. All the drugs in this study with wide variety of pK_a values were well trapped by this column under slight acidic condition, and high recovery of each drug was obtained by using two kinds of elution solvents, TFA and ammonia in ACN solutions.

Fig. 2 shows the mass chromatogram of the extract from human plasma spiked with 100 ng/mL each of 43 drugs and IS. Forty-three drugs were well separated from each other by using $[M+H]^+$ ion from 7-aminonitrazepam to 2-oxoquazepam within 12 min (Fig. 2, Table 1).

Table 2 shows calibration range and correlation coefficients, within- and between-day precision (RSD, %), recovery (%) and limit of detection (ng/mL) of each drug. The calibration curve was liner in the concentration range shown in Table 2 at each drug with correlation coefficients exceeding 0.99. Within-day precisions (RSD, %) of this method were 1.8–15.6% (10 ng/mL) and 0.6–10.1% (100 ng/mL) and between-day precisions (RSD, %) were 1.6–16.9% (10 ng/mL) and 0.6–16.7% (100 ng/mL).

The recoveries of drugs using the Focus™ column at 100 ng/mL were more than 70% in 36 drugs, 50–70% in 6 drugs, and less than 50% in 1 drug (average 87.1%). The low recovery of 7-aminonitrazepam (31.1%) was probably due to weak affinity of the drug to Focus™ column. The recoveries of drugs at 10 ng/mL tended to be lower (average 70.1%). Very high recoveries (>120%) observed in some samples containing low concentrations of 2-hydroxyethylflurazepam and ethyl lofrazepate may derive from poor separation from components of matrix.

The LOD values ranged from 0.2 to 8.0 ng/mL in 37 drugs and signal-to-noise ratio was more than 3 in 6 drugs even at the lowest level we examined (0.2 ng/mL). Ion suppression by the matrix was lower than 10% for all drugs at the level of both 10 and 100 ng/mL, respectively except for 7-aminonitrazepam (80%). Since the same amount of ion suppression (80%) were observed for 7-aminonitrazepam in 3 different sources of plasma, the method could be acceptable for this drug as long as the same matrix is used for making a calibration curve.

UPLC/MS with Acquity UPLC column could allow high speed, high resolution and high sensitivity even with scan mode. The particle size in Acquity UPLC column is 1.7 μm which is small compared with that for conventional LC (e.g., 3.5 or 5.0 μm). It is thought that the smaller particle size decreases the height equivalent to a theoretical plate (HETP), thus separation efficiency is improved, according to van Deemter plot [15]. This column also contains cross ethylene bridged structure between internal particles which provides high-pressure resistance (up to 15,000 psi) to flow through small particles with high density.

We combined scan mode with SIR mode in one run in order to analyze all drugs simultaneously. Zopiclone, zolpidem, flurazepam, clonazepam, triazolam, blotizolam and lormetazepam were measured by SIR mode since therapeutic concentrations of these drugs in plasma were sub ng/mL order [22,23] or intensity was low. It is desirable that unknown samples are analyzed with scan mode in order to find the intoxicated drugs. But high sensitivity is certainly obtained with SIR mode. Furthermore, many analytical time windows are required when only SIR mode was selected for the analysis of all 43 drugs, resulting in the insufficient number of data point in each peak. And it will be impossible to detect compounds in tight time windows when retention time shift occurs by column contamination. Therefore, we carefully selected drugs and minimized drug numbers for SIR mode measurement. Thus we could develop a simultaneous quantification method for 43 drugs in combination with scan mode and SIR mode without loss of the number of data point in each peak.

Although further confirmation may be required by direct comparison of mass spectrum pattern with respective standard substance using higher cone voltage in order to avoid false positive result [24–25], and additional deuterium-labeled ISs may give more accurate quantification value for some drugs, our method was considered to be sufficiently useful as the first screening and rough estimation of drug level in the sample.

4. Practical application

The established method has been applied to not only plasma samples but also whole blood and urine samples in forensic cases,

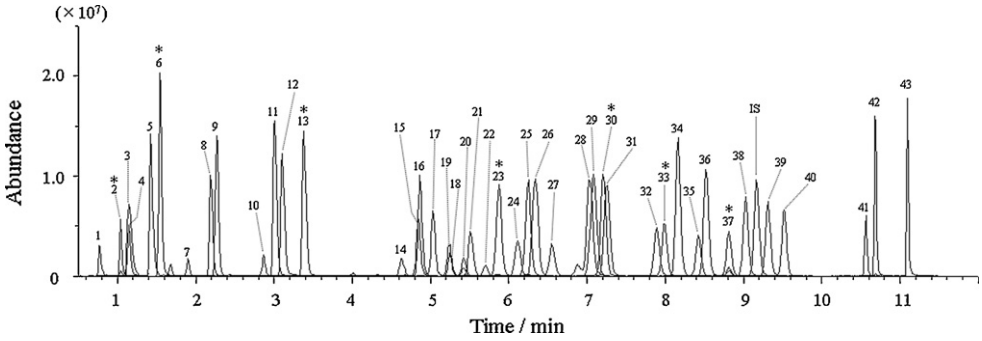

Fig. 2. Mass chromatogram of the extract from human plasma spiked with 100 ng/mL each of 43 drugs and IS. *These drugs were measured with SIR mode in UPLC/MS analysis.

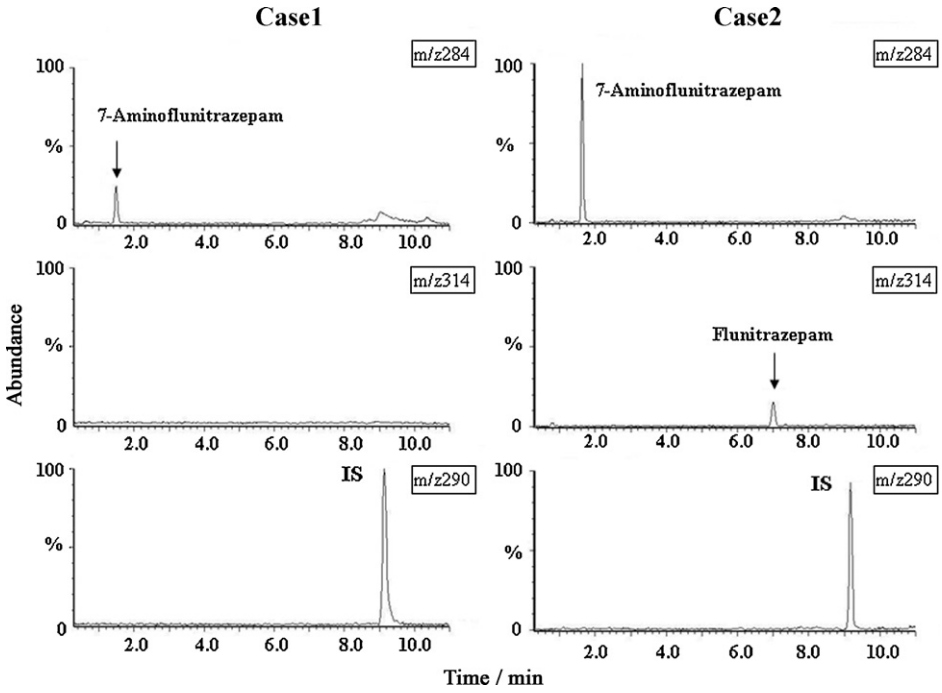

Fig. 3. Mass chromatograms of the extracts from the whole blood of two autopsy cases.

and given reliable data so far. Fig. 3 shows the mass chromatograms of the extracts from the whole blood of two autopsy cases where ingestion of flunitrazepam was confirmed. Flunitrazepam was given before strangulation in case 1, and the drug was ingested for suicide attempt in case 2. Only 7-aminoflunitrazepam was detected in case 1 with the concentration of 26.7 ng/mL, and both flunitrazepam and 7-aminoflunitrazepam were detected in case 2 with the concentrations of 84.2 and 567.1 ng/mL, respectively. Therefore, our method using UPLC/MS proved to be useful in forensic practice.

5. Conclusion

We developed a rapid and highly sensitive screening method for 43 benzodiazepines, their metabolites, zolpidem and zopiclone in human plasma using UPLC/MS with combined scan and SIR mode. This method will be useful in the analyses of forensic and clinical toxicological cases.

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