



A new method for high-resolution and high-precision analysis of flunitrazepam and 7-aminoflunitrazepam in human body fluids using a Monolithic SPE SpinTip and UPLC–Q-ToF–MS

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Received: 10 December 2018 / Accepted: 5 March 2019 / Published online: 20 March 2019
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Abstract

Purpose Forensic toxicological analyses of drugs and their metabolites in human specimens usually require extractive pretreatment for successful analysis of substances from the matrix. In the present study, a high-throughput method was developed to analyze flunitrazepam, 7-aminoflunitrazepam, 7-acetamidoflunitrazepam, 7-acetamido-3-hydroxyflunitrazepam, and 3-hydroxyflunitrazepam in human plasma and urine samples using a new Monolithic C₁₈ gel-packed SpinTip and ultra-performance liquid chromatography (UPLC)–quadrupole time-of-flight (Q-ToF) mass spectrometry (MS).

Methods Plasma (20 µL) or urine (100 µL) samples spiked with each component were extracted using a Monolithic C₁₈ SPE SpinTip and quantified by UPLC–Q-ToF–MS with positive-ion electrospray ionization (ESI).

Results Good separation, with clear peak shapes of flunitrazepam and its metabolites, was achieved within an analysis time of 6 min, including the extraction time. Recoveries of flunitrazepam and 7-aminoflunitrazepam for plasma and urine samples were 93.5–118% and 97.7–109%, respectively. The regression equations for flunitrazepam and 7-aminoflunitrazepam showed excellent linearity in the range of 0.5–250 ng/mL for plasma and 0.4–500 ng/mL for urine, with detection limits of 0.2–0.5 ng/mL. Intra- and inter-day coefficients of variations for two drugs are smaller than 13.5%. The accuracy of quantitation was 89–110%.

Conclusions The method was successfully applied to determine the level of flunitrazepam and its metabolites in human plasma and urine, respectively, after oral administration to a volunteer.

Keywords Flunitrazepam · Metabolites · Monolithic C₁₈ SPE SpinTip · UPLC–Q-ToF–MS

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11419-019-00471-4>) contains supplementary material, which is available to authorized users.

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Introduction

Benzodiazepines are widely used as sedatives, anxiolytics, and hypnotics. These drugs are also frequently encountered in emergency toxicology screening, drug abuse testing, and forensic medical examinations [1–4]. Flunitrazepam (Rohypnol[®]), 5-(2-fluorophenyl)-1-methyl-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one, is a typical benzodiazepine fast-acting hypnotic drug, and has attracted attention as a notorious “party drug” or “date rape drug,” with serious social implications [4–7]. Therefore, methods for determining the concentration of flunitrazepam in human samples are needed for diagnosis and effective treatment of intoxication and for forensic purposes.

Generally, therapeutic drug monitoring (TDM) of flunitrazepam or its abuse has been performed by detecting its metabolites such as 7-aminoflunitrazepam,

3-hydroxyflunitrazepam, 7-acetamidoflunitrazepam, and 7-acetamido-3-hydroxyflunitrazepam. 7-Aminoflunitrazepam is the most important and major metabolite of flunitrazepam, which can persist for several days in human blood and urine after drug intake. One of the required analytical features is the sensitivity to determine very low concentrations of flunitrazepam and its metabolites in human samples [4, 5]. Accurate and rapid methods for detecting and measuring unchanged flunitrazepam and/or its metabolites in human samples, therefore, are required both for diagnosis and effective treatment of intoxication and for forensic purposes.

Several methods have been reported for determining the levels of flunitrazepam and its metabolites in various matrices using gas chromatography (GC)–mass spectrometry (MS) [8, 9] or tandem MS (MS/MS) [10, 11] and high-performance liquid chromatography (HPLC)–MS [12, 13] or HPLC–MS/MS [14–17]. In order to eliminate sample impurities present in human body fluids, most of these analytical techniques employ extraction steps such as liquid–liquid extraction (LLE) [12, 14–16], solid-phase extraction (SPE) [9, 11, 13, 17], or liquid-phase microextraction (LPME) [8, 10]. Although LLE and SPE can successfully extract drugs from biological fluids, these procedures are generally performed in an offline mode and are usually labor-intensive and time-consuming. Moreover, traditional methods typically involve the use of large amounts of volatile compounds. The frequent use of toxic organic solvents can cause problems with regard to health and the environment. In recent years, SPE micropipette tips such as ZipTip [18, 19] and MonoTip [20–23] have been used as suitable tools for the purification, concentration, and selective isolation of biological samples to remedy these issues. However, because of manual operation, there are problems such as occurrence of SPE tip clogging or uneven extraction efficiency, especially for biological fluids [18–23]. Generally, pre-analytical hydrolysis by enzymatic or chemical protocols have been used to pretreatment of urine samples [8, 17]. In this study, those glucuronide and/or sulfate conjugates can not be targets for emergency analysis because of these techniques are complicated and time-consuming.

We developed a unique Monolithic silica SPE technique for sample preparation using SpinTips, which offered simpler, faster, and higher-throughput extractions than techniques using conventional SPE tips or cartridges. Monolithic silica (2.8 mm i.d. × 1 mm thickness), consisting of continuous mesoporous (10 nm) silica skeletons ~10 µm in size and 5-µm through-pores, was fixed into a 200-µL pipette tip (Fig. S1). The Monolithic silica surface was modified with a C₁₈ phase. The small bed volume and sorbent mass within such tips allowed for reduced solvent and elution volumes, reduced extraction times, and high throughput.

HPLC–MS/MS that is based mainly on triple quadrupole instruments is currently the most widely applied system for analyzing biological samples [14–17]. During the operation in a multiple reaction monitoring mode, HPLC–MS/MS systems obtain reliable quantitative information. However, the required qualitative information to support the structural elucidation of analytes is poor. Recently, ultra-performance liquid chromatography (UPLC)–quadrupole time-of-flight (Q-ToF)–MS has been used for the unequivocal confirmation of compounds from biological and environmental samples by accurate mass measurements of protonated and deprotonated molecules [24–28]. Accurate mass measurements of MS/MS product ions have also become particularly important in structural elucidation of unknowns. Indeed, the use of Q-TOF–MS shows important benefits through the use of mass accuracy full-scan spectral libraries or databases, making the identification and quantification of both target compounds and unknowns feasible with a high degree of confidence.

In the present study, we established a high-throughput, reproducible, and practical procedure for analyzing flunitrazepam and its metabolites in human plasma and urine samples using Monolithic C₁₈ SPE SpinTip extraction and UPLC–Q-ToF–MS analysis. To the best of our knowledge, this is the first report dealing with Monolithic C₁₈ SPE SpinTip for extracting flunitrazepam from human body fluid samples.

Materials and methods

Materials

Flunitrazepam was provided by Tatsumi Kagaku Co., Ltd. (Kanazawa, Japan). 7-Aminoflunitrazepam, flunitrazepam-*d*₇ (internal standard, IS), and 7-aminoflunitrazepam-*d*₇ (IS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HPLC–MS-grade methanol was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other common chemicals used were of the highest purity and were available commercially. Ultra-pure water from the Milli-Q ultra-pure system (Komatsu Electronics Co., Ltd., Ishikawa, Japan) was used in all experiments. Monolithic C₁₈ SPE SpinTips (C₁₈-bonded monolithic silica gel with a diameter of 2.8 mm, thickness of 1 mm, weight of 2.5 mg, mesopore size of 10 nm, through-pore size of 5 µm, and surface area of 350 m²/g) were provided from GL Sciences (Tokyo, Japan).

Preparation of plasma and urine samples

Drug-free whole blood and urine samples were obtained from healthy volunteers recruited from among laboratory personnel. To prepare drug-free plasma samples, the heparinized whole blood was centrifuged at 1700×g for

10 min at 4 °C, and the plasma was decanted into a clean centrifuge tube. These obtained drug-free plasma and urine samples were stored at –80 °C until use.

Preparation of standard solutions and quality control samples

Individual stock standard solutions (1 mg/mL) of flunitrazepam, 7-aminoflunitrazepam, and the two ISs were prepared separately by dissolving an accurately weighed quantity of each drug in methanol. The solutions were then stored at 4 °C. Working standard solutions of these drugs were prepared by appropriate dilution of the stock standard solutions using the HPLC mobile phase (50% aqueous methanol with 0.1% formic acid). All working standard solutions were freshly prepared every week and stored at 4 °C. Calibration standards were prepared by mixing appropriate amounts of working standard solutions and drug-free plasma and urine to achieve eight different concentrations ranging from 0.5 to 250 ng/mL (0.5, 1.25, 2.5, 5.0, 25.0, 50, 100, and 250 ng/mL) and 50 ng/mL each of two ISs for plasma, as well as 0.4–500 ng/mL (0.4, 1.0, 2.5, 5.0, 25.0, 50, 200, and 500 ng/mL) and 10 ng/mL each of two ISs for urine. Quality control (QC) samples (0.4–500 ng/mL) for flunitrazepam and 7-aminoflunitrazepam were also prepared using the same procedure.

Extraction procedure using the Monolithic C₁₈ SPE SpinTip

Flunitrazepam, 7-aminoflunitrazepam, and two ISs were extracted from human plasma and urine using a Monolithic C₁₈ SPE SpinTip. Briefly, the SpinTip was inserted into a spin adapter placed in a microcentrifuge polypropylene tube (1.5 mL), conditioned with 100 µL methanol, and centrifuged at 1000×g for 15 s, followed by 100 µL of ultra-pure water at 1000×g for 15 s. For human plasma samples, 170 µL of ultra-pure water was added to 20 µL of the plasma containing 10 µL of drug mixture (flunitrazepam, 7-aminoflunitrazepam, and two ISs). For human urine samples of 100 µL containing 10 µL of drug mixture (flunitrazepam, 7-aminoflunitrazepam, and two ISs), 10 µL of 1 N HCl solution and 80 µL of ultra-pure water were added. The sample solutions were applied to the conditioned Monolithic C₁₈ SPE SpinTip and centrifuged at 1000×g for 15 s. The SPE SpinTip was then washed with 100 µL of ultra-pure water at 1000×g for 15 s, and the analytes were eluted from the SPE SpinTip with 50 µL of methanol at 1000×g for 10 s. A 5-µL aliquot of the eluate was directly analyzed by UPLC–Q-ToF–MS.

UPLC–Q-ToF–MS conditions

A UPLC–Q-ToF–MS system consisting of an Acquity UPLC liquid chromatograph (Waters, Milford, MA, USA) and a Xevo G2 Q-TOF mass spectrometer (Waters) was used for all measurements.

The Waters Acquity UPLC system was equipped with a binary solvent manager, a sample manager, and a column oven. The chromatographic separation of the flunitrazepam and its metabolites, and two ISs was achieved on a Waters Acquity UPLC BEH C₁₈ column (50 mm × 2.1 mm i.d., particle size 1.7 µm) with a linear gradient elution system composed of 0.1% aqueous formic acid solution (pH 2.8) and methanol at a flow rate of 0.4 mL/min. Solvent A was 0.1% formic acid in ultra-pure water (v/v), and solvent B was methanol. Gradient runs were programmed to change from 90% solvent A/10% solvent B to 40% solvent A/60% solvent B within 3.5 min. The column was subsequently maintained with 1% solvent A/99% solvent B for 1.0 min and then re-equilibrated with 90% solvent A/10% solvent B for 0.5 min before the next injection. The total chromatographic run time was 5 min.

For confirmation, mass spectrometry measurements were performed using a Xevo G2 Q-TOF mass spectrometer. The analyses were carried out using the electrospray ionization (ESI) setting in a positive mode. The mass spectrometer was operated in a positive ion mode, with a capillary voltage of 3.0 kV and a cone voltage of 30 V. The source and desolvation temperatures were 150 °C and 500 °C, respectively. The desolvation gas flow and the cone gas flow were 1000 and 50 L/h (both N₂), respectively. The considered mass range was 100–1000 Da. Data were collected in centroid mode, with the sensitivity analyzer mode selected. The accuracy and reproducibility of all analyses were guaranteed using a LockSpray. Leucine-enkephalin was used as the lock mass at a concentration of 1 ng/mL in 50% aqueous acetonitrile with 0.1% formic acid and a flow rate of 5 µL/min. MassLynx version 4.1 (Waters) was used to analyze the samples, with the following parameter settings: analysis time of 0–4 min, spectrum above the relative intensity of 2%, and maximum tolerance of mass error set as 5 ppm. The prediction rules of elemental composition (EC) were defined as follows: atom numbers of carbon, hydrogen, oxygen, nitrogen, and fluorine were set to ranges of 0–100, 0–200, 0–20, 0–20, and 0–6, respectively. The molecular formula assignments were obtained with the MassLynx i-FIT algorithm. For flunitrazepam, 7-aminoflunitrazepam, and two ISs, the search was restricted to molecules containing CHONF only, and the best fit was obtained on both mass accuracy and isotope intensity pattern (i-FIT). Blank human plasma or urine samples were used as controls for comparison with the analytical samples, and all were processed under the same conditions.

Method validation

The method was validated for linearity, selectivity, precision, accuracy, matrix effect, and recovery according to the US Food and Drug Administration guidelines for bio-analytical method validation [29]. Regression equations for flunitrazepam and 7-aminoflunitrazepam were obtained by plotting the peak-area ratio of analytes/IS (*y*-axis) against the analyte concentration (*x*-axis). The slope and *y*-intercept of the regression line were estimated in duplicate for each of eight different calibrations and on six consecutive days. The acceptance criterion for the correlation coefficient is >0.999 . The limit of detection (LOD, $s/n=3$) was obtained by measuring the signal-to-noise ratio of blank plasma or urine spiked with the lowest concentration of each analyte. The lower limit of quantification (LLOQ, $s/n=10$) was obtained by measuring the signal-to-noise ratio of blank plasma or urine spiked with the lowest concentration on the calibration curve of each analyte.

The selectivity of the method was estimated by analyzing blank human plasma and urine matrix samples. The responses of the interfering substances or background noises at the retention time of flunitrazepam, 7-aminoflunitrazepam, and two ISs were acceptable if they were less than 5% of the mean response of the LLOQ. Intra- and inter-day precision and accuracy were carried out by analyzing QC samples spiked with flunitrazepam and 7-aminoflunitrazepam at three different concentrations (0.4, 1.0, 1.25, 2.0, 5.0, 20, 50, 100, 200, 250, or 500 ng/mL) in six replicate samples on the same day. The concentration of analytes in the QC samples was calculated using the calibration curves. The precision was determined by calculating the coefficient of variation (CV), whereas the accuracy was expressed as a percentage of the mean of the measured concentration against the nominal concentration. The evaluations of precision were based on previously published criteria [29]. The accepted criterion for precision (percentage CV) is $\leq 15\%$. Matrix effect and recovery was calculated by comparing the chromatographic peak areas of the analyte in QC samples with those obtained by direct injection of analyte standards dissolved in methanol, and determined at different concentration levels (0.4, 1.0, 1.25, 2.0, 5.0, 20, 50, 100, 200, 250, or 500 ng/mL).

Administration of flunitrazepam to a healthy volunteer

The present method was applied to real samples of human plasma and urine to confirm its utility. After obtaining his informed consent, a therapeutic dose of flunitrazepam (1 mg) was administered orally to a 52-year-old male volunteer (body weight, 73 kg) at 8 a.m. after meals. The whole blood (10 mL) and urine (20 mL) samples were collected pre-dose (0 h) and 0.5, 1, 2, 8, 10, 12, 24, 48, and 60 h

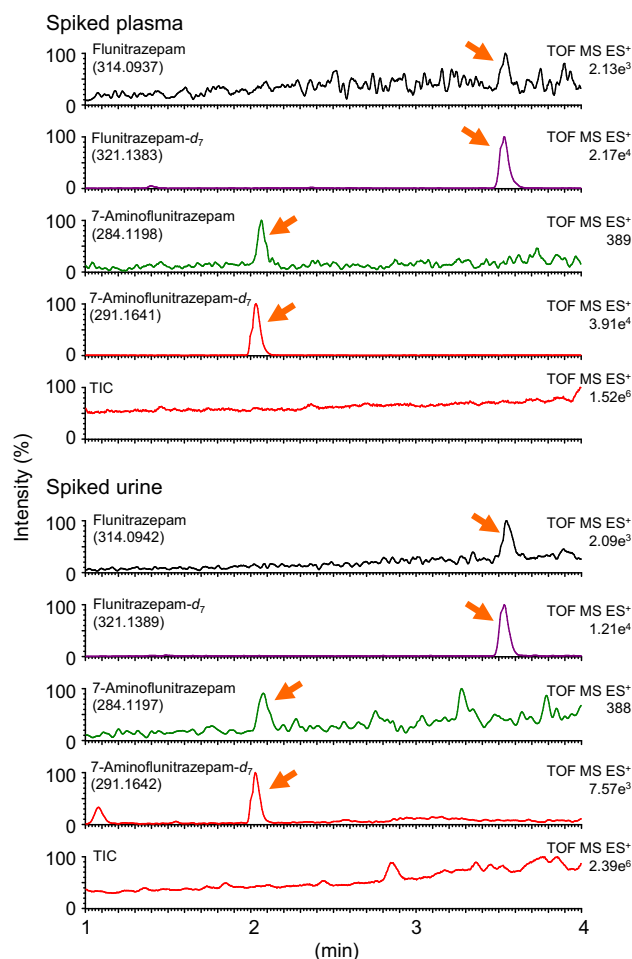


Fig. 1 TIC and XIC of UPLC-Q-ToF-MS for flunitrazepam, 7-aminoflunitrazepam, and two ISs from human plasma and urine in positive ESI mode. Drug-free plasma samples (20 μ L) or urine samples (100 μ L) were spiked test compounds at concentration of LLOQ for flunitrazepam and 7-aminoflunitrazepam, and 50 ng/mL or 10 ng/mL each of two ISs for plasma and urine, respectively. The MS spectra of XIC are consistent with those reported in Table 1

after drug administration and transferred to centrifuge tubes containing heparin sodium. The heparinized blood samples were centrifuged at $1700\times g$ for 10 min at 4 °C. The resulting plasma and urine samples were stored at -80 °C until analysis.

Results and discussion

Optimization of extraction conditions for the Monolithic C₁₈ SPE SpinTip

Various types of SPE tips packed with functionalized monolithic silica are commercially available (Supplemental Fig. S1 and Table S1). They can exhibit reversed-phase, normal-phase, or ion-exchange adsorption capacity; some feature

Table 1 Mass spectral data for flunitrazepam, 7-aminoflunitrazepam, and ISs from human plasma and urine

Compound	Molecular formula [M+H] ⁺	Theoretical <i>m/z</i>	Experimental <i>m/z</i>	Error	
				mDa	ppm
Plasma					
Flunitrazepam	C ₁₆ H ₁₃ N ₃ O ₃ F	314.0941	314.0937	−0.4	−1.3
Flunitrazepam- <i>d</i> ₇ (IS)	C ₁₆ H ₆ 2H ₇ N ₃ O ₃ F	321.1380	321.1383	0.3	0.9
7-Aminoflunitrazepam	C ₁₆ H ₁₅ N ₃ OF	284.1199	284.1198	−0.1	−0.4
7-Aminoflunitrazepam- <i>d</i> ₇ (IS)	C ₁₆ H ₈ 2H ₇ N ₃ OF	291.1639	291.1641	0.2	0.7
Urine					
Flunitrazepam	C ₁₆ H ₁₃ N ₃ O ₃ F	314.0941	314.0942	0.1	0.3
Flunitrazepam- <i>d</i> ₇ (IS)	C ₁₆ H ₆ 2H ₇ N ₃ O ₃ F	321.1380	321.1389	0.9	2.8
7-Aminoflunitrazepam	C ₁₆ H ₁₅ N ₃ OF	284.1199	284.1197	−0.2	−0.7
7-Aminoflunitrazepam- <i>d</i> ₇ (IS)	C ₁₆ H ₈ 2H ₇ N ₃ OF	291.1639	291.1642	0.3	1.0

Identification analysis was carried out using MassLynx 4.1 software. Drug-free plasma (20 μ L) or urine (100 μ L) samples were spiked with the test compounds at concentrations of LLOQ for flunitrazepam and 7-aminoflunitrazepam, and 50 ng/mL or 10 ng/mL each of two ISs for plasma and urine, respectively

Table 2 Correlation coefficient, LOD, and LLOQ for flunitrazepam and 7-aminoflunitrazepam extracted from human fluids

Compound	Correlation ^a coefficient (<i>r</i>)	Concentration range (ng/mL)	LOD ^b (ng/mL)	LLOQ ^c (ng/mL)
Plasma				
Flunitrazepam	0.9995	1.25–250	0.5	1.25
7-Aminoflunitrazepam	0.9990	0.5–100	0.2	0.5
Urine				
Flunitrazepam	0.9996	1.0–500	0.5	1.0
7-Aminoflunitrazepam	0.9996	0.4–200	0.2	0.4

^aThe data were subjected to linear regression analysis of peak area ratios (*y*) of a compound to IS (50 ng/mL each for plasma and 10 ng/mL each for urine) against the spiking concentrations (*x*). Eight plots (each point represents the mean of duplicate determinations) with different concentrations for each drug were used

^bThe limit of detection (LOD) is measuring the signal-to-noise ratio at *s/n* = 3

^cThe lower limit of quantification (LLOQ) is measuring the signal-to-noise ratio at *s/n* = 10

titanic ligands. The surface area of monolithic silica gel is greater than that for MonoTip, which is the same as MonoSpin. Consequently, the main advantages of Monolithic C₁₈ SPE SpinTips are that trap capacity is much higher than that of the MonoTip, and almost no clogging occurs (Table S1). For determination of blood concentrations of a target compound in biological samples, whole blood, plasma, or serum is usually used. When these specimens are used in undiluted form, clogging of tips can easily occur. Thus, in the present study, plasma and urine samples were diluted tenfold and twofold with ultra-pure water, respectively.

For the SpinTip SPE, the sample solution is passed through the tips by centrifugation. For the best extraction of target compounds by Monolithic C₁₈ SPE SpinTips, the centrifugal speed during sample loading should be optimized. In the preliminary experiments, the optimal relative centrifugal force (RCF) and centrifugation time was checked using Monolithic C₁₈ SPE SpinTips. However, to achieve sufficient

reproducibility that did not cause clogging of the Monolithic C₁₈ SPE SpinTips, a minimum RCF and centrifugation time of 1000 \times *g* for 15 s was chosen for sample loading. Moreover, to obtain good efficiency and high purity for these target compounds, preparation of the sample solution composition to suit ligand bound to silica skeleton (pH adjustment) is critical. For extraction of flunitrazepam and its metabolite, 7-aminoflunitrazepam, using Monolithic C₁₈ SPE SpinTips, pH 6.9 and 2.4 gave the best results for plasma and urine samples, respectively (Fig. S2).

The entire Monolithic C₁₈ SPE SpinTip extraction process required approximately 2 min, including conditioning, sample loading, washing, and elution. In contrast, the time required to manually perform conventional cartridge SPE exceeds 20 min [30–33]. In addition, the eluate from the Monolithic C₁₈ SPE SpinTips could be injected directly into the UPLC–Q-ToF–MS instrument without evaporation and reconstitution steps, which is particularly important for

Table 3 Precision and accuracy for flunitrazepam and 7-aminoflunitrazepam in QC samples

Compound	Concentration added (ng/mL)	Intra-day (<i>n</i> = 6)			Inter-day (<i>n</i> = 6)		
		Concentration detected (ng/mL)	Precision (CV, %)	Accuracy (%)	Concentration detected (ng/mL)	Precision (CV, %)	Accuracy (%)
Plasma							
Flunitrazepam	5	4.90 ± 0.17 ^a	3.5	98.0	4.95 ± 0.10	2.0	99.0
	50	49.5 ± 0.6	1.2	99.0	48.3 ± 2.2	4.6	96.6
	250	254 ± 1.9	0.7	102	254 ± 3.5	1.4	102
7-Aminoflunitrazepam	2	1.93 ± 0.07	3.6	96.5	1.90 ± 0.12	6.3	95.0
	20	18.5 ± 0.2	1.1	92.5	18.2 ± 0.6	3.3	91.0
	100	101 ± 1.3	1.3	101	101 ± 2.0	2.0	101
Urine							
Flunitrazepam	1	0.97 ± 0.04	4.1	97.0	0.89 ± 0.12	13.5	89.0
	50	50.9 ± 1.2	2.4	102	51.3 ± 1.2	2.3	103
	500	497 ± 6.8	1.4	99.4	513 ± 15.2	3.0	103
7-Aminoflunitrazepam	0.4	0.44 ± 0.05	11.4	110	0.43 ± 0.05	11.6	108
	20	20.6 ± 0.5	2.4	103	20.8 ± 0.9	4.3	104
	200	199 ± 3.6	1.8	99.5	201 ± 10.7	5.3	101

CV coefficient of variation

^aThe values are mean ± SD

Table 4 Matrix effect, recovery, and their coefficients of intra-day variation (CV) for flunitrazepam and 7-aminoflunitrazepam from human plasma and urine samples

Compound	Amount added (ng/mL)	Matrix effect (%)	Recovery (%)	CV (%)
Plasma				
Flunitrazepam	5	6.5	93.5 ± 1.7	1.8
	50	3.7	102 ± 1.1	1.1
	250	4.9	105 ± 0.4	0.4
7-Aminoflunitrazepam	2	3.6	98.0 ± 1.6	1.6
	20	9.2	108 ± 1.2	1.1
	100	16.4	118 ± 1.6	1.4
Urine				
Flunitrazepam	1	5.7	97.7 ± 6.4	6.6
	50	4.3	102 ± 6.9	6.8
	500	3.8	101 ± 4.3	4.3
7-Aminoflunitrazepam	0.4	10.1	109 ± 6.7	6.1
	20	7.7	107 ± 5.5	5.1
	200	8.4	107 ± 9.2	8.6

The values are mean ± SD of four experiments. Efficiency was calculated by comparing the peak areas obtained from the pre-extracted spiked specimens with those obtained from the pre-extracted spiked initial mobile phase using the present method

rapid and simple analyses. Therefore, the use of Monolithic C₁₈ SPE SpinTips is recommended for rapid extraction of flunitrazepam and 7-aminoflunitrazepam from human body fluids. The total solvent volume used for each step of the extraction process was 150 µL, which is lower than the volumes required for conventional SPE cartridges (around 1.5–65 mL) [30–33]. Furthermore, the required plasma or urine sample volume was reduced to 20 or 100 µL, respectively, which corresponds to 5–100 times less than volumes previously reported for flunitrazepam analysis in plasma and urine samples [8–17]. Traditionally, pre-analytical hydrolysis by enzymatic or chemical protocols has been used for pretreatment of urine samples [8, 17]. However, these techniques are complicated and time-consuming. In contrast, small amounts of non-conjugated forms of flunitrazepam and 7-aminoflunitrazepam can be detected using the present method, and it is much simpler with higher throughput than the hydrolysis procedures. Such small volumes of solvent and samples needed in the method used in this study represent a significant advance in sample preparation miniaturization. The overall procedure can be considered “green” because it requires little solvent and produces little waste.

Mass spectra

Figure 1 shows total ion chromatogram (TIC) and extracted ion chromatogram (XIC) obtained by UPLC–Q–ToF–MS for these drugs from human plasma and urine containing the test compounds at concentrations of LLOQ for flunitrazepam and 7-aminoflunitrazepam, and 50 ng/mL or 10 ng/mL each

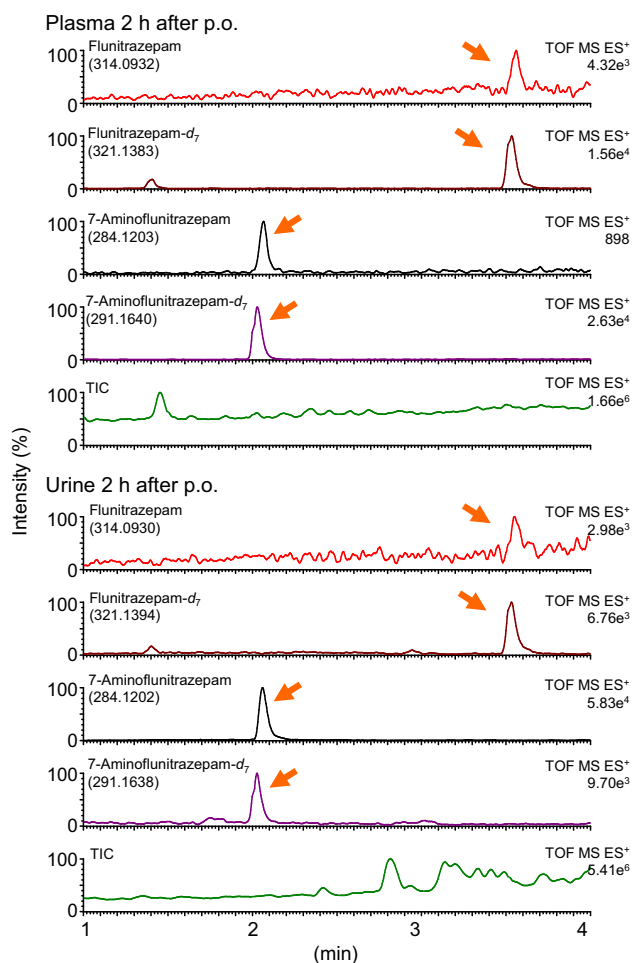


Fig. 2 TIC and XIC of UPLC–Q–ToF–MS from human plasma and urine 2 h after oral administration of flunitrazepam (1 mg). The amount of flunitrazepam-*d*₇ and 7-aminoflunitrazepam-*d*₇ used as ISs were 50 ng/mL each of plasma and 10 ng/mL each of urine, respectively

of two ISs for plasma and urine, respectively. Distinct peaks appeared on the chromatograms within 4 min for each drug and the two ISs. Blank chromatograms gave small impurity peaks, and no interfering peaks appeared around the retention times of the test compounds (data not shown). The obtained spectra showed accurate corresponding masses for the deprotonated molecular ions. Table 1 shows the mass spectral data for flunitrazepam, 7-aminoflunitrazepam, and two ISs obtained by UPLC–Q–ToF–MS using a positive ESI mode. Flunitrazepam, flunitrazepam-*d*₇, 7-aminoflunitrazepam, and 7-aminoflunitrazepam-*d*₇ detected in a positive-ion mode gave the protonated molecule $[M+H]^+$ at m/z 314.0937, 321.1383, 284.1198, and 291.1641 for plasma and m/z 314.0942, 321.1389, 284.1197, and 291.1642 for urine, respectively, in the full-scan mode (Table 1). These

protonated molecules were identified as $C_{16}H_{13}N_3O_3F$ (–1.3 ppm mass error, 0.057 i-FIT) for flunitrazepam, $C_{16}H_6_2H_7N_3O_3F$ (0.9 ppm mass error, 0.006 i-FIT) for flunitrazepam-*d*₇, $C_{16}H_{15}N_3OF$ (–0.4 ppm mass error, 0.001 i-FIT) for 7-aminoflunitrazepam, $C_{16}H_8_2H_7N_3OF$ (0.7 ppm mass error, 0.002 i-FIT) for 7-aminoflunitrazepam-*d*₇ from plasma, $C_{16}H_{13}N_3O_3F$ (0.3 ppm mass error, 0.134 i-FIT) for flunitrazepam, $C_{16}H_6_2H_7N_3O_3F$ (2.8 ppm mass error, 0.039 i-FIT) for flunitrazepam-*d*₇, $C_{16}H_{15}N_3OF$ (–0.7 ppm mass error, 0.241 i-FIT) for 7-aminoflunitrazepam, and $C_{16}H_8_2H_7N_3OF$ (1.0 ppm mass error, 0.005 i-FIT) for 7-aminoflunitrazepam-*d*₇ from urine, respectively (Table 1).

Method performance

The regression equations for flunitrazepam and 7-aminoflunitrazepam gave good linearity for both plasma and urine samples, with correlation coefficients of at least 0.9990 (Table 2). The present method also showed good linearity for the known components tested. The LOD and LLOQ values for flunitrazepam and 7-aminoflunitrazepam under optimal conditions were 1.25–250 ng/mL and 0.5–100 ng/mL in plasma, and 1.0–500 ng/mL and 0.4–200 ng/mL in urine, respectively. The therapeutic levels of flunitrazepam and 7-aminoflunitrazepam were reported to be 1.5–15 ng/mL [34–37] and 0.8–3.0 ng/mL [36, 37] in whole blood, serum or plasma, and 1.2–1.4 ng/mL [36, 37] and 20–143 ng/mL [37] in urine, respectively. For autopsy cases, the high concentrations of flunitrazepam and 7-aminoflunitrazepam in postmortem specimens have been reported to be 4–750 ng/mL and 5–7100 ng/mL, respectively [14, 37–40].

Intra- and inter-day precision and accuracy was evaluated by assessing QC samples prepared from human plasma and urine (Table 3). The intra- and inter-day CVs were no greater than 13.5%, and the accuracy ranged from 89 to 110% for all concentrations, leading us to consider the variability acceptable for method validation based on the current criteria [41, 42]. The percentage matrix effect values obtained at three different concentrations are shown in Table 4, and were determined as 3.7–6.5% for flunitrazepam and 3.6–16.4% for 7-aminoflunitrazepam in human plasma and urine samples, respectively. The signal deviations for both compounds were less than 16%. Furthermore, the matrix effect did not cause quantification bias, as evidenced by the intra-day CV values of 0.4–8.6% for flunitrazepam and 7-aminoflunitrazepam. This variability was considered acceptable for validation of the method based on current criteria [41, 42]. It was concluded that the matrix effect was not a significant issue for this method. The recoveries of flunitrazepam and 7-aminoflunitrazepam from human plasma and urine samples were determined at three different concentrations ranging from

Table 5 Concentrations of flunitrazepam and its metabolite, 7-aminoflunitrazepam, in plasma and urine samples for up to 60 h after oral administration of flunitrazepam (1 mg) in a healthy volunteer

Time (h)	Concentration (ng/mL)			
	Plasma		Urine	
	Flunitrazepam	7-Aminoflunitrazepam	Flunitrazepam	7-Aminoflunitrazepam
0.5	8.40 ^a ± 1.68	0.63 ± 0.05	<LLOQ ^d	<LLOQ ^e
1	15.2 ± 0.75	1.08 ± 0.10	1.83 ± 0.49	42.5 ± 5.45
2	11.4 ± 1.64	1.58 ± 0.05	2.38 ± 1.36	76.3 ± 6.63
8	4.60 ± 1.40	1.38 ± 0.13		
10			<LLOQ	102 ± 2.75
12	3.05 ± 0.90	1.48 ± 0.10	<LLOQ	39.9 ± 1.99
24	<LLOQ ^b	<LLOQ ^c	<LLOQ	35.7 ± 0.72
48				13.1 ± 0.39
60				6.55 ± 0.49

^aThe values are mean ± SD (*n* = 4)

^bLower limit of quantification (LLOQ) is 1.25 ng/mL

^cLower limit of quantification (LLOQ) is 0.5 ng/mL

^dLower limit of quantification (LLOQ) is 1.0 ng/mL

^eLower limit of quantification (LLOQ) is 0.4 ng/mL

93.5 to 118% (Table 4). The increased or reduced recovery is probably due to a combination of the decrease or increase of analyte during sample preparation steps and ion suppression/enhancement from the UPLC–Q-ToF–MS analysis step.

Actual measurements of flunitrazepam and its metabolites in human plasma and urine after oral administration

In addition to analyses of spiked human plasma and urine, the present method was applied to human plasma and urine samples from a male volunteer. Figure 2 shows UPLC–Q-ToF–MS XICs for human plasma and urine 2 h after oral administration, respectively. The drug concentrations of flunitrazepam and 7-aminoflunitrazepam in plasma and urine after the administration of flunitrazepam calculated by internal calibration are shown in Table 5. Peak concentrations of flunitrazepam were obtained after 1 h from plasma (15.2 ng/mL) and urine (1.83 ng/mL) samples and were still positive after 12 h for plasma and 2 h for urine samples. 7-Aminoflunitrazepam was present from 0.5 to 12 h in plasma and 1–60 h in urine samples, with the highest concentration after 2 h (1.58 ng/mL) for plasma and 10 h for urine (102 ng/mL).

Moreover, other widely known metabolites for flunitrazepam such as 3-hydroxyflunitrazepam, 7-acetamidoflunitrazepam, and 7-acetamido-3-hydroxyflunitrazepam together with 7-aminoflunitrazepam were estimated using the present method from human urine 12 h after oral administration.

Figure 3 and Table S2 show the XIC and mass spectral data for four metabolites and 7-aminoflunitrazepam-*d*₇ (IS) obtained by the present UPLC–Q-ToF–MS using a positive ESI mode. Those XICs were generated using the theoretical mass value with a ± 5 ppm extraction window. The deprotonated molecules are assigned as C₁₆H₁₃N₃O₄F (1.2 ppm mass error, 0.261 i-FIT) for 3-hydroxyflunitrazepam, C₁₈H₁₇N₃O₂F (0.6 ppm mass error, 0.309 i-FIT) for 7-acetamidoflunitrazepam, C₁₈H₁₇N₃O₃F (1.2 ppm mass error, 0.128 i-FIT) for 7-acetamido-3-hydroxyflunitrazepam, and C₁₆H₁₅N₃O₄F (1.1 ppm mass error, 0.019 i-FIT) for 7-aminoflunitrazepam.

Conclusions

We have established a detailed and novel procedure for the quantitative determination and identification of flunitrazepam and its metabolites in human body fluid samples using a Monolithic C₁₈ SPE SpinTip and UPLC–Q-ToF–MS analysis. Compared to LLE and conventional SPE, the present SpinTip SPE technique reduced sample extraction time, solvent consumption, and clogging of SPE, and enhanced ease of operation. Furthermore, the present SpinTip SPE technique required only a small volume (20–100 µL) of sample to accomplish the analysis, which is extremely useful when the available sample volume is small. Under optimized conditions, good recovery, linearity, and reproducibility were obtained. The method was successfully applied

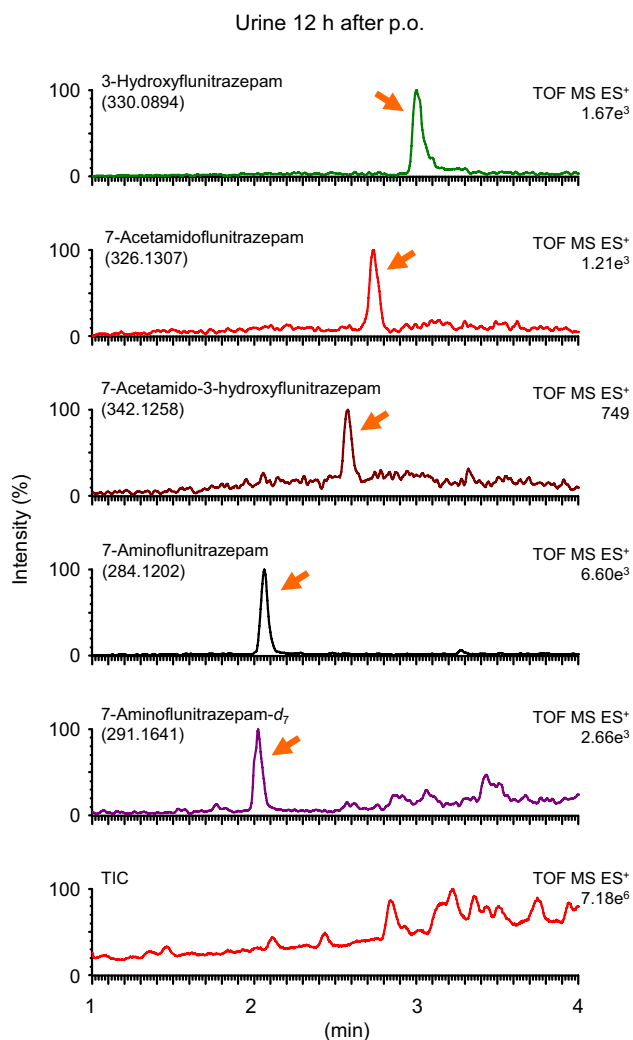


Fig. 3 TIC and XIC of UPLC–Q–ToF–MS from human urine 12 h after oral administration of flunitrazepam (1 mg). The amount of 7-aminoflunitrazepam- d_7 used as IS was 10 ng/mL of urine

to actual human plasma and urine samples collected from a volunteer after oral administration. It was also very useful as a preliminary (pilot) method for the screening and quantitative determination of flunitrazepam and its metabolites in clinical and toxicological analyses. The procedure can be easily modified and expanded to encompass other drugs, if necessary.

Acknowledgements This study was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), KAKENHI Grant (C) 24590865.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest associated with this manuscript.

Ethical approval This study was approved by the Ethics Committee of Showa University School of Medicine (no. 861). The obtainment of blood and urine samples from healthy volunteers was approved by the Ethics Committees of Showa University School of Medicine (no. 1249).

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