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LETTER TO THE EDITOR

Investigation on toxicological usefulness of synovial fluids, as an alternative matrix; postmortem distribution/redistribution of triazolam and its predominant metabolite α-hydroxytriazolam in human body fluids

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Dear Editor,

Benzodiazepine class drugs are commonly prescribed throughout the world [1]. Because those compounds generally have pharmacological affinities to γ-aminobutyric acid (GABA) receptors at synaptic membrane in human brain, benzodiazepines can bring suppressive effects to central nervous system. In addition, they also have muscle-relaxing effects in human body. Thus, various benzodiazepines have been prescribed for treatment of insomnia, and also used for anti-epileptic, anti-convulsant, sedative and hypnotic effects as medicine, according to their differences in elimination half-life and potencies. Especially in forensic area, benzodiazepines have also been one of the most encountered drugs in many cases involved in unexpected and sudden deaths [2], suicidal [3], homicidal [4] and drug facilitated crime (DCF) cases [5,6].

Among various types of benzodiazepine, triazolam (8-chloro-6-(o-chlorophenyl)-1-methyl-4*H*-s-triazolo [4,3a][1,4] benzodiazepine) have been used as one of the most potent short acting drugs for treatment of insomnia and as sedatives in pre-anesthetic medical cares. Its major metabolite 1-hydroxytriazolam (α-hydroxytriazolam) is also estimated to be at least as active as parent triazolam, which can reach about 50 to 100% of activity of triazolam [7]; in contrast, another metabolite 4-hydroxytriazolam has a reduced activity [7]. Like other benzodiazepines, numerous

triazolam related cases have also been reported in forensic toxicological area so far [8-10]. Various human specimens including body fluids, organs and tissues are used as biological specimens for investigating involved compounds. Especially as fluidal specimens, in addition to blood and urine, various kinds of specimen including pericardial fluid, cerebrospinal fluid (CSF) and synovial fluid (SF) can also be collected and tested as alternative matrices in forensic toxicological interpretations [11-14]. SF is biological lubricant existing in almost all articular joints, components of which consists of blood plasma, proteins and various biochemical compounds [15]. As alternative specimen in forensic toxicological investigations, SFs have also been used for detecting methanol, ethanol and certain drugs such as opiate and cocaine [16-19]. However, the studies on distribution of involved drugs and/or its metabolites in SF specimens in intoxication cases are sporadic, and thus of the very limited number. Recently, we experienced a suicidal case, wherein triazolam could contribute to the cause of death. In this article, we present the postmortem distribution/redistribution of triazolam and its predominant metabolite α-hydroxytriazolam in SF, as an alternative forensic specimen, along right heart blood, femoral vein blood, CFS and pericardial fluid and bile specimens obtained from the same suicidal case. Although a couple of reports on postmortem distribution of triazolam and its predominant metabolite α-

hydroxytriazolam among various human samples have been published so far [9,20], to our best knowledge, the postmortem distribution/redistribution of triazolam and its metabolite in SF has not been reported.

A female in her 80s was found dead by her family, in her bed room. The very near to the body, about 80 empty blister packages of triazolam (0.125 mg) tablets, equivalent to be about 10 mg of triazolam, were also found. The triazolam tablets were thought to be ingested prior to her death. Therefore, the police first suspected that she had died of drug poisoning due to triazolam, as manner of suicide; it was disclosed that she had been diagnosed as insomnia prior to the death and triazolam had been prescribed for her, with no criminal evidences being found through investigation.

A forensic autopsy for the victim was performed at our department. The postmortem interval was estimated to be about 3 days after her death. The female victim was 155 cm high and weighed 63.6 kg. Postmortem rigidity was relatively marked at all joints. Remarkable lividity accompanied by vibices was found on her face and back. As external findings, small petechial hemorrhages were observed at conjunctivae of the right and left eyelid. Upon internal findings, a lot of white froth were observed inside the airway. Both lungs were slightly congested, with the right and left lungs being 440 and 337 g, respectively. Almost all organs including the brain were also congested. As a

remarkable finding, inside the stomach, there were 135 g light brown contents including a piece of thin metal, which derived from the blister packages of triazolam; neither intact tablets nor its residue could be found in the stomach. Except for the above findings, there were neither pathological findings nor injuries by macroscopic observation. Analysis of blood alcohol by gas–chromatography showed negative results for right heart blood specimen. Immunochemical drug screening kit Triage Drugs of Abuse panel (Alere, Waltham, MA, USA) for the stomach contents showed positive result for the benzodiazepine group; there was almost no urine in the bladder, and thus we could not obtain a sufficient volume of urine specimen for the screening kit. Therefore, considering above autopsy findings, the direct cause of her death was suspected to be triazolam poisoning.

Triazolam was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan); α-hydroxytriazolam from Sigma-Aldrich (St. Louis, MO, USA); triazolam-d_4 and α-hydroxytriazolam-d_4 from Cerilliant (Round Rock, TX, USA) as internal standards (ISs); β-glucuronidase (100 kU/mL) from Kura Biotech (Puerto Varas, Chile). Other common chemicals used were of the highest purity commercially available. The QuEChERS dispersive solid-phase extraction (SPE) centrifuge tubes with caps (2-mL capacity), each of which contained 25 mg of primary-secondary amine (PSA), 25 mg

of end-capped octadecylsilane (C_{18EC}) and 150 mg of magnesium sulfate, and Captiva ND Lipids cartridges (3-mL capacity) were purchased from Agilent (Santa Clara, CA, USA). Whole blood specimens from the femoral vein and the right atria in heart, bile, pericardial fluid, CSF, stomach contents and SFs from right and left knee joints were obtained from the deceased at autopsy, and kept frozen at -80°C until analysis; each SF specimen could be collected to be about 1.0 mL and more from left and right knee joint, respectively, using needle with glass syringe.

Extraction procedure was as followed; each 100-μL volume of body fluid was added to 0.9-mL of acetonitrile, followed by mixing with 10 ng of ISs (triazolam-d_4 and α-hydroxytriazolam-d_4) dissolved in each 10-μL of acetonitrile. To the mixture, various amounts (0, 5, 10, 50, 100 and 500 ng) of triazolam and α-hydroxytriazolam was also added for determination by the standard addition method [21]. Then, each mixture was shaken gently and centrifuged at 3000 rpm for 10 min in a plastic test tube. Then, the supernatant was decanted into a QuEChERS SPE centrifuge tube, vortexed for 30 s, and centrifuged again at 3000 rpm for 10 min. Each 600-μL volume of upper acetonitrile layer was passed through a Captiva ND Lipids cartridge. A 3.5-μL aliquot of the eluate was then analyzed by the below liquid chromatography－tandem mass spectrometry (LC－MS/MS).

Hydrolysis procedure was as followed; each 100-μL volume of body fluid was adjusted to pH 5 with 100-μL 1 M acetic acid buffer and incubated with β-glucuronidase (100 kU/mL) at 37 ℃ for 2 h. After hydrolysis, 0.8-mL acetonitrile was added to the mixture and centrifuged at 3000 rpm for 10 min. The subsequent procedure leading to LC–MS/MS was the same as described above.

LC－MS/MS with electrospray ionization (ESI) was conducted on an Agilent 1200 LC-SL system connected to a 6460 Triple Quad LC/MS tandem MS instrument (Agilent). The Agilent LC-SL system included a microdegasser and a high-performance autosampler. For LC separation, a ZORBAX Eclipse Plus C18 column (100 × 2.1 mm i.d., particle size 1.8 μm; Agilent) was used. The LC conditions were: injection volume, 3.5 μL; flow rate, 0.2 mL/min; elution mode, gradient with 10 mM ammonium formate/0.1% formic acid in distilled water (A) and acetonitrile (B) from 50% A/50% B to 100% B over 10 min, followed by post-running elution with the initial solvent composition for 10 min. The separation column was operated under room temperature, which was controlled around 28°C; autosampler was operated at 4°C. The tandem MS condition were: interface, ESI mode; polarity, positive ion mode; ion source temperature, 320°C; ion source voltage, 500 V; quantification, selected reaction monitoring (SRM) mode using the peak area; ion transitions, *m/z* 343 → 308 for triazolam, *m/z 359* → 239

for α-hydroxytriazolam, *m/z* 347 → 312 for triazolam-d_4 and *m/z 363* → 243 for α-hydroxytriazolam-d_4; fragmentor voltage and collision energy were 25 and 140 V for triazolam, 40 and 170 V for α-hydroxytriazolam, 13 and 170 V for triazolam-d_4, and 37 and 170 V for α-hydroxytriazolam-d_4, respectively.

Under the conditions, target compounds could successfully be separated in all samples examined; the retention times of triazolam, α-hydroxytriazolam, triazolam-d_4 and α-hydroxytriazolam-d_4 were 3.87, 2.75, 3.79 and 2.74 min, respectively. As shown in Figs. 1 and 2, all peaks appeared sufficiently sharp and symmetric; background levels were generally low, and there were no impurity peaks which interfered with the target and IS peaks.

Product ion mass spectra of the reference standard triazolam, α-hydroxy triazolam, extracts of femoral vein blood, SF, and other fluidal specimens tested were obtained by LC－MS/MS. The profiles of product ion mass spectra obtained from these specimens coincided well with those of the reference standard triazolam and α-hydroxytriazolam, without additional impurity peaks. Thus, it was confirmed that triazolam and its metabolite were both involved in all specimens examined in our analysis.

The standard addition method [21] was used to measure concentrations of triazolam and α-hydroxtriazolam in different human body fluids in this study. Recently,

standard addition method is frequently employed for quantification of target compounds in forensic toxicological field [22-25]. This method can overcome the differences in matrix effects and even recovery rates according to kinds of specimens in our studies (e.g., simultaneous determinations of target compound(s) in body fluids and solid tissues). In addition, the method requires no matrix-matched blanks without target compound(s). The detailed procedure and the calculation method for quantification were described in our previous literature [21]. The correlation coefficient values of the standard addition calibration equations for triazolam and α-hydroxytriazolam for all specimens examined were not smaller than 0.99. The lower quantification limit (signal-to-noise ratio $\geqq$ 10) was around 1.0 ng/mL.

Because the standard addition method was employed in this study without the use of blank specimens, the usual accuracy and precision data for validation could not be presented. Instead, as shown in Table 1, each intraday and interday repeatability of triazolam and α-hydroxytriazolam in SF and femoral vein blood specimens was examined. The repeatability value, expressed as relative standard deviations, was not greater than 8.8% for all specimens tested, showing that repeatabilities in this method were satisfactory.

In this study, QuEChERS dispersive solid-phase extraction coupled with a Captiva ND Lipids cartridge was employed for extractions of target compounds. Slight suppressive matrix effects, not lower than 83.6%, were found in analysis for triazolam and α-hydroxytriazolam in both SF and femoral vein blood specimens examined, showing that matrix effects of them for analysis were relatively slight (Table 2). After compensation calculation of suppressive matrix effects on triazolam and α-hydroxytriazolam in the SF specimens, the recovery rate values of them were 76.8 and 76.6%, respectively (Table 2). For the femoral vein blood specimens, those of triazolam and α-hydroxytriazolam were 101 and 97.9%, respectively (Table 2). It was noted that recovery rate values on SF were slightly lower than those in femoral vein blood specimens, which suggested that difference in components between the whole blood and SF could influence recoveries of target compounds by extraction procedure for specimens employed in this analysis.

Stabilities of the triazolam and α-hydroxytriazolam in both SF and femoral vein blood specimens were assessed as followed; a couple of SF and femoral vein blood specimens at 100 ng/mL of triazolam or α-hydroxytriazolam were prepared, and left at room temperature (28℃) or stored at -80℃ (used as 100% control) in freezer for seven days. Then, extraction procedure followed by LC－MS/MS analysis was conducted for

each specimen. The peak areas of triazolam and α-hydroxytriazolam in SF and femoral vein blood specimens left under room temperature were found to be slightly lower (< 10%) than those stored in the deep freezer. Therefore, it was shown that stabilities of them under tested conditions could provide few influences on results of analysis.

Table 3 shows the postmortem distribution of triazolam and α-hydroxytriazolam in right and left SF, CSF, pericardial fluid, right heart blood, femoral vein blood, bile and stomach content specimens obtained from the same victim; concentrations of α-hydroxytriazolam in tested specimens, those of before and after hydrolyzations by β-glucuronidase were also shown in the same table. Concentration of triazolam in right and left SF, right heart and femoral vein blood specimens of this case were 22.1, 20.7, 78.3 and 38.5 ng/mL, respectively. The triazolam concentration in right heart blood was found to be higher than that in femoral vein blood. It was estimated that the difference between concentrations in femoral vein blood and right heart blood could be due to postmortem distribution/redistribution of triazolam; the loss of concentration of triazolam by diffusion across the thin wall of peripheral femoral vein also could contribute to the difference [27].

According to previous studies [8], blood concentration levels of triazolam in fatal cases (triazolam alone poisoning) were reported to range from 10 to 153 ng/mL. The collected data of toxic blood concentrations for many drugs [28] showed that comatose-

fatal blood concentration was 30 ng/mL for triazolam. Therefore, blood concentration of triazolam in our case was thought to be consistent with fatal intoxication by triazolam ingestion.

In this case, the concentrations of triazolam and its predominant metabolite α-hydroxytriazolam in SF specimens could also be detected and successfully quantified. Concentrations of triazolam in SF specimens were determined to be slightly lower than those in blood specimens; on the other hand, α-hydroxytriazolam concentration was found to be about two times higher than those in femoral vein blood specimen after hydrolysis. Regarding α-hydroxytriazolam in SF specimens, its concentration was markedly increased between before and after hydrolysis, showing about as much as 10 times increase by hydrolysis; remarkable increase of α-hydroxytriazolam by hydrolysis in femoral vein blood specimens could not be observed in this case. The unique glucuronidation of α-hydroxytriazolam should be confirmed again. If it is correct, it will be of interest to examine whether such accumulation of conjugated metabolite in SF can take place for other benzodiazepines.

Finally, it should be mentioned that this is the first report to show the distribution of triazolam and its predominant metabolite α-hydroxytriazolam in SF, as an alternative specimen to be detected by LC–MS/MS.

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Declarations

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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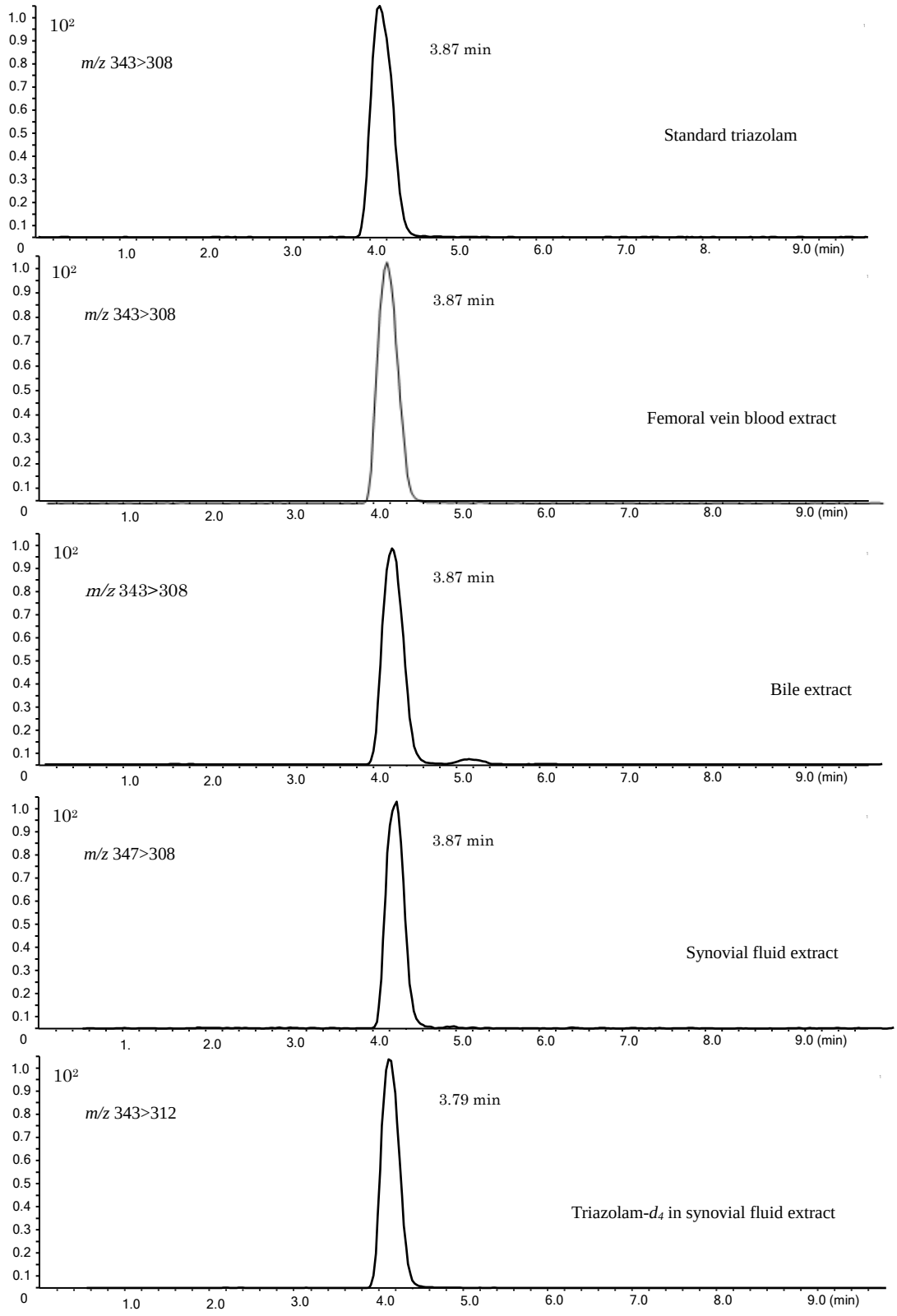
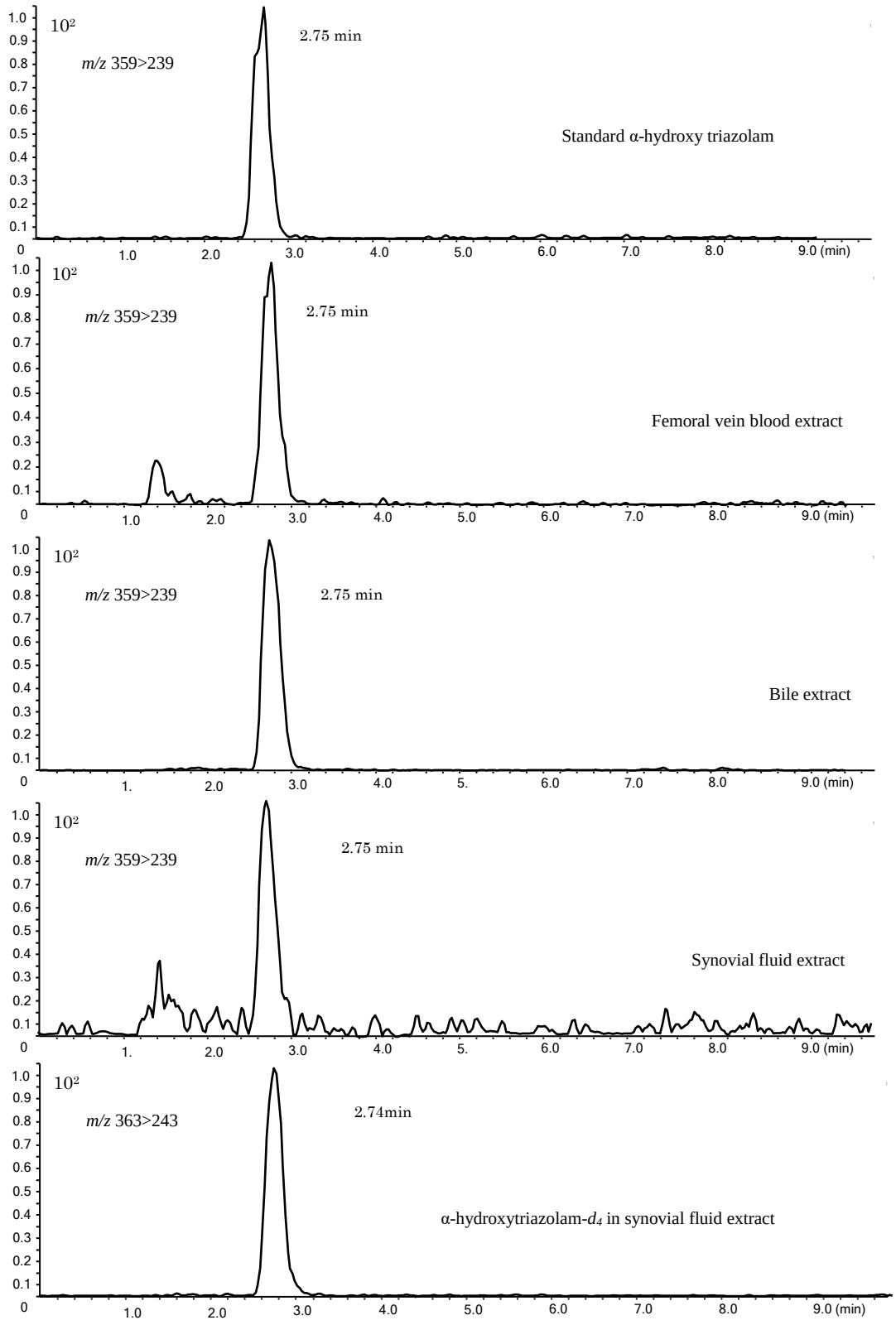

10²
m/z 343>308
3.87 min
Standard triazolam
10²
m/z 343>308
3.87 min
Femoral vein blood extract
10²
m/z 343>308
3.87 min
Bile extract
10²
m/z 347>308
3.87 min
Synovial fluid extract
10²
m/z 343>312
3.79 min
Triazolam-d4 in synovial fluid extract
1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 (min)

Fig. 1


10²
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2.75 min
Standard α-hydroxy triazolam
10²
m/z 359>239
2.75 min
Femoral vein blood extract
10²
m/z 359>239
2.75 min
Bile extract
10²
m/z 359>239
2.75 min
Synovial fluid extract
10²
m/z 363>243
2.74min
α-hydroxytriazolam-d4 in synovial fluid extract
1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 (min)

Fig. 2

Figure legends

Fig. 1 Selected reaction monitoring (SRM) chromatograms using LC–MS/MS for reference standard of triazolam and the extracts of the tested synovial fluid samples. In the bottom panel, the chromatogram for the IS spiked into synovial fluid is also presented

Fig. 2 SRM chromatograms using LC–MS/MS for reference standard of α-hydroxytriazolam and the extracts of the tested samples. In the bottom panel, the chromatogram for the IS spiked into synovial fluid is also presented

Table 1 Intraday and interday repeatability for determination of triazolam and α-hydroxytriazolam in synovial fluid and femoral vein blood specimens

Analyte	Specimen	Intraday (n = 5)		Interday (n = 5)	
		Concentration (ng/mL)	Repeatability (%RSD)	Concentration (ng/mL)	Repeatability (%RSD)
Triazolam	Left synovial fluid	20.7 ± 1.8	8.8	20.9 ± 1.3	6.5
	Femoral vein blood	38.5 ± 0.9	2.5	37.4 ± 3.2	8.7
α-hydroxytriazolam	Left synovial fluid	53.6 ± 2.9	5.4	55.0 ± 2.6	4.7
	Femoral vein blood	28.3 ± 1.5	5.3	29.1 ± 2.4	8.2

RSD relative standard deviation

Table 2 Matrix effects and recovery rates of triazolam and α-hydroxytriazolam in synovial fluid and femoral vein blood specimens under our procedure

Analyte	Specimen	Matrix effect (%)	Recovery (%)
Triazolam	Left synovial fluid	87.9±1.8	76.8±6.4
	Femoral vein blood	87.1±2.4	101±5.6
	Bile	92.2±6.2	99.7±3.2
α-Hydroxytriazolam	Left synovial fluid	92.8±4.3	76.6±8.6
	Femoral vein blood	83.6±4.7	98.9±4.4
	Bile	92.5±2.1	112±7.5

Table 3 Concentrations of triazolam and α-hydroxytriazolam in examined specimens of the deceased

Specimen	Concentration (ng/mL, mean ± STD, *n* = 3)		
	Triazolam	Free α-Hydroxytriazolam	α-Hydroxytriazolam (after hydrolysis)
Right synovial fluid	22.1 ± 0.8	5.6 ± 0.2	55.8 ± 4.2
Left synovial fluid	20.7 ± 1.8	4.9 ± 0.3	-[a]
Femoral vein blood	38.5 ± 0.9	25.3 ± 1.5	28.8 ± 3.3
Right heart blood	78.3 ± 1.0	31.5 ± 0.6	-[a]
Cerebrospinal fluid	23.5 ± 0.7	7.5 ± 0.2	20.0 ± 1.3
Pericardial fluid	121 ± 2.8	44.4 ± 1.0	47.0 ± 4.7
Bile	184 ± 8.6	135 ± 2.6	2036 ± 210
Stomach contents	365 ± 32.5	40.9 ± 2.9	-[a]

STD standard deviation

[a] not available