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Application News

No. C183

Liquid Chromatography Mass Spectrometry

Simultaneous Analysis of Etizolam, Triazolam, and Their Metabolites in Biospecimens Using LCMS™-9030

Introduction

Etizolam and Triazolam are psychotropic drugs in the benzodiazepine class, which are used for a wide range of purposes due to their properties as a sedative, hypnotic drug, anxiolytic, anticonvulsant, and muscle relaxant. Benzodiazepines and their metabolites in the body are important analytes in the field of forensic toxicology and therefore more reliable toxicology test methods, such as liquid chromatography mass spectrometry (LC/MS), are needed.

Since Etizolam, Triazolam, and their metabolites (alpha-Hydroxyetizolam, alpha-Hydroxytriazolam, and 4-Hydroxytriazolam) have similar structures (Fig. 1) or almost the same molecular weight, it is difficult to separate them using high performance liquid chromatography (HPLC) or by the difference in *m/z* values that can be detected by a quadrupole mass analyzer with nominal mass resolving power. In this experiment, we simultaneously analyzed Etizolam, Triazolam, and their metabolites using the Nexera™ X2 ultra high performance liquid chromatograph and LCMS-9030 high-resolution mass spectrometer.

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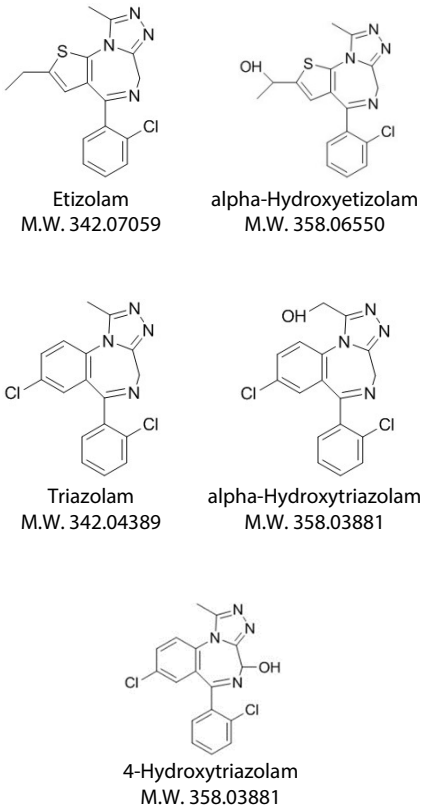

Fig. 1 Molecular Structures of Etizolam, Triazolam, and Their Metabolites

Instrument and Analysis Conditions

The analysis conditions of HPLC and the mass spectrometer are given in Table 1. As the HPLC conditions, a column packed with 1.9 µm particles was selected for the purpose of separating the metabolites. As for the mobile phases, 0.1 % formic acid solution and acetonitrile (containing 0.1 % formic acid) were used.

Table 1 Analysis Conditions

HPLC Conditions		
System	Nexera X2	
Column	YMC-Triart C18 (1.9 µm, 50 × 2 mm)	
Mobile phase A	0.1 % formic acid + water	
Mobile phase B	0.1 % formic acid + acetonitrile	
Flow rate	0.6 mL/min	
Time program	Time (min)	Concentration of B (%)
	0.00	20
	3.00	20
	11.50	30
	11.51	95
	13.00	95
	13.01	20
	15.00	STOP
Injection volume	2 µL	
Column temperature	40 °C	
MS Conditions		
System	LCMS-9030	
Ionization method	ESI (+)	
Nebulizer gas flow rate	3.0 L/min	
Heating gas flow rate	10.0 L/min	
Drying gas flow rate	5.0 L/min	
Interface temperature	300 °C	
DL temperature	250 °C	
Heat block temperature	400 °C	

Pretreatment of Spiked Blood and Plasma Samples

A volume of 100 mg of Q-sep™ QuEChERS extraction salt packet (Q150 packet, Restek) was placed in a 2.0 mL microtube as packing materials, to which three ϕ 3 mm stainless steel beads, 300 µL of acetonitrile, 200 µL of distilled water, standard substances of each compound, and 100 µL of human whole blood or blood plasma were added. Followed by centrifugal separation (10,000 rpm, 10 min), the supernatant was collected as a sample. The standards were added to matrices to achieve a concentration equivalent to 10 ng/mL and a spike and recovery test was performed. The pretreatment workflow is shown in Fig. 2.

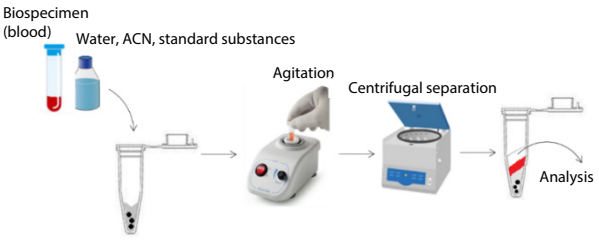

Fig. 2 Pretreatment Workflow Diagram

Experiment Results

Fig. 3 shows an extracted-ion chromatogram (XIC) (extraction range: theoretical *m/z* value ± 2 mDa of each compound) obtained from the mixture of Etizolam, Triazolam, and their metabolites (50 ng/mL). Compounds with a difference of 26 mDa, such as Etizolam and Triazolam or alpha-Hydroxyetizolam and alpha-Hydroxytriazolam, were detected from peaks at different retention times. Etizolam and Triazolam were sufficiently separated from their retention times. Alpha-Hydroxytriazolam and alpha-Hydroxyetizolam, which cannot be sufficiently separated by HPLC, were measured individually to check the level of separation by accurate mass. The mass range was individually set for each XIC.

When alpha-Hydroxyetizolam was measured individually, a peak was detected only on the XIC with the following extraction range: the theoretical *m/z* value ± 2 mDa (*m/z*: 359.0708 to 359.0748) of the target compound. A peak was not detected on the XIC with an extraction range of *m/z* from 359.0441 to 359.0481 (figures on the left side of Fig. 4). In addition, as with the case of alpha-Hydroxyetizolam, a peak was detected only on the XIC with an extraction range of the theoretical *m/z* value ± 2 mDa (*m/z*: 359.0441 to 359.0481) when measured individually (figures on the right side of Fig. 4).

The peaks of alpha-Hydroxytriazolam and alpha-Hydroxyetizolam overlap each other because their retention times are nearly the same; however, the results indicate that they can be selectively quantified without interference from the other compound by using an XIC with the extraction range of the theoretical *m/z* value ± 2 mDa for the detection of each compound.

Fig. 5 shows the calibration curves created from the standard samples of Etizolam, Triazolam, and their metabolites. The results of spike and recovery tests performed by adding each standard to a whole blood or blood plasma sample at a concentration equivalent to 10 ng/mL are shown in Table 2. Each psychotropic drug and metabolite was successfully detected at a sufficient level of sensitivity at each concentration. Furthermore, the coefficient of determination of the calibration curve of all compounds reached 0.999, indicating an excellent linearity of the results. Also, good quantitative results were obtained from the matrices of the psychotropic drugs and their metabolites using these calibration curves: the range was 105 % to 115 % for whole blood samples and 117 % to 131 % for blood plasma samples.

The mass chromatograms of blank samples, whole blood and plasma samples to which the psychotropic drugs and their metabolites were spiked are shown in Fig. 6. As shown by the chromatograms, these compounds were detected only from the whole blood and plasma samples and were not detected from blank samples. In addition, alpha-Hydroxytriazolam and 4-Hydroxytriazolam in the matrix were separated by using liquid chromatography (LC).

Relative mass errors of standard solutions and matrices at each concentration are given in Table 3. The concentration hardly affected the relative mass errors and the results ranged from –0.229 mDa to 0.335 mDa, which were extremely good. The relative mass error range of whole blood and plasma samples was –0.270 mDa to 0.354 mDa, which confirmed that the range of relative mass errors of matrices is also stable.

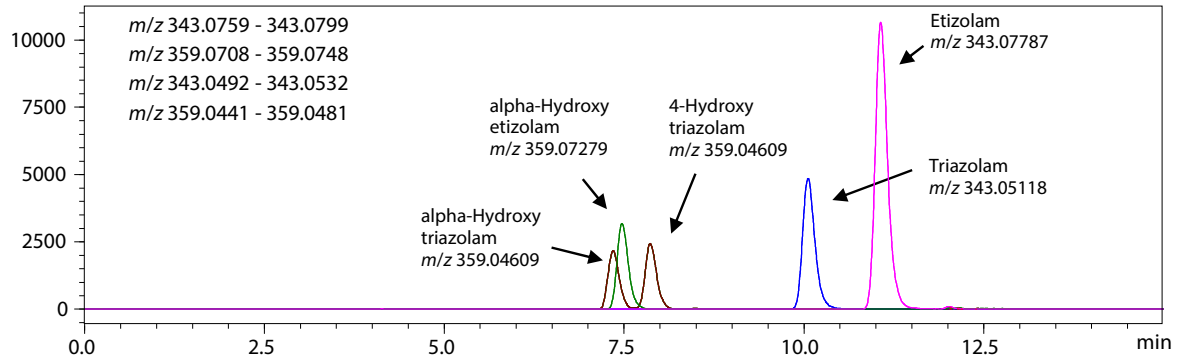

Fig. 3 Mass Chromatogram (Extraction Range: Theoretical Value ± 2 mDa) of Etizolam, Triazolam, and Their Metabolites (50 ng/mL)

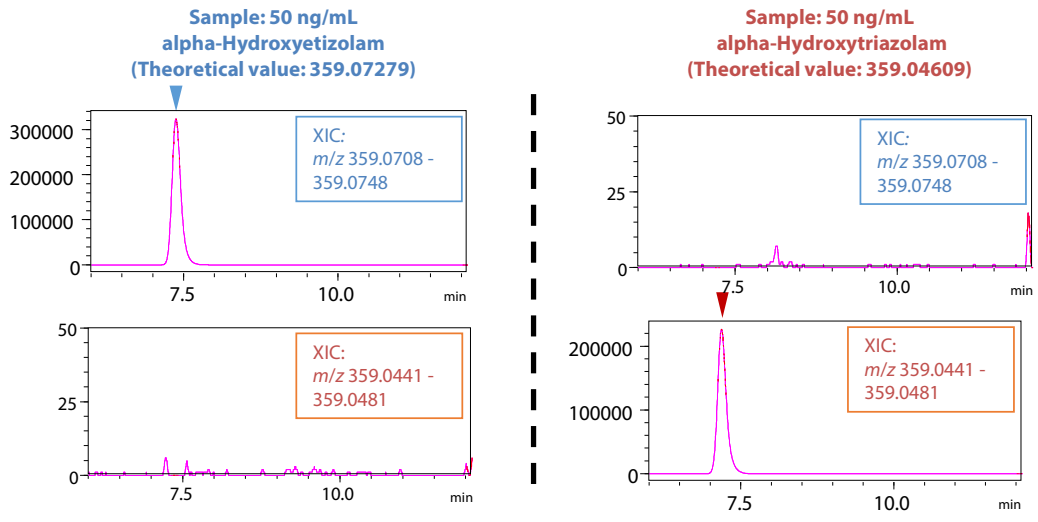

Fig. 4 Separation of alpha-Hydroxyetizolam and alpha-Hydroxytriazolam by Mass Resolution

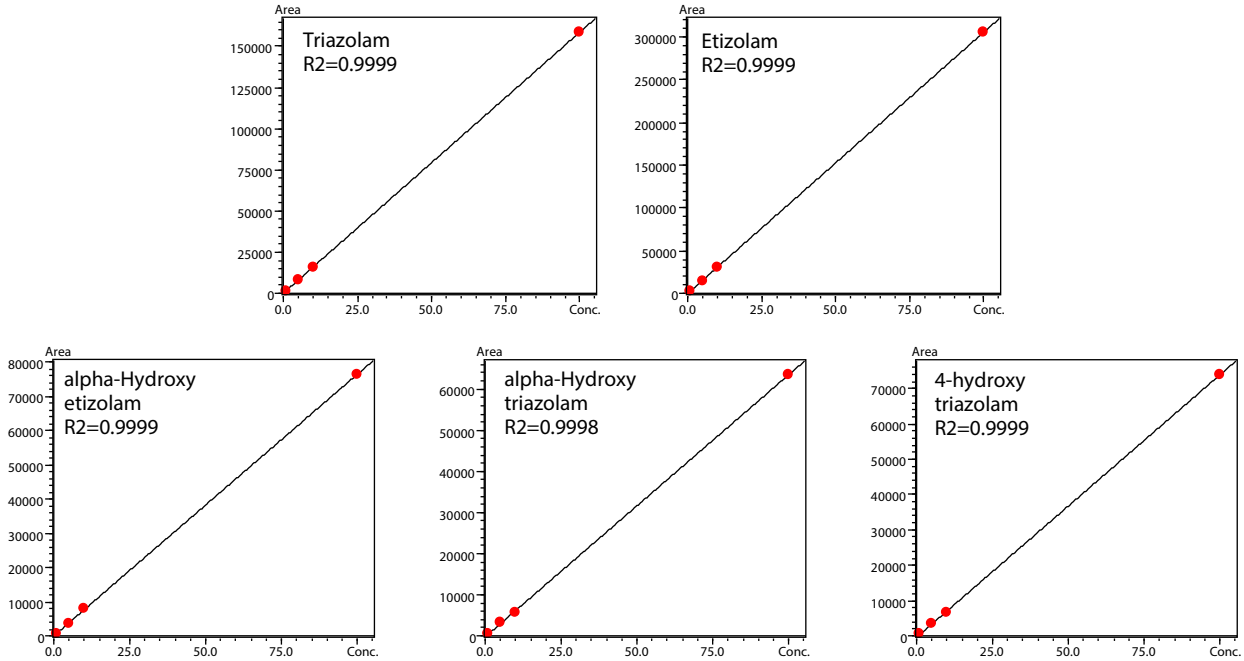

Fig. 5 Calibration Curves of Each Compound (1 to 100 ng/mL)

Table 2 Quantitative Results of Each Compound

Compound Name	Spike Concentration (ng/mL)					
	Standard Solution				Whole Blood	Blood Plasma
	1	5	10	100	10	10
	Accuracy (%)					
Etizolam	107	96	102	100	105	117
alpha-Hydroxy etizolam	92	97	103	100	105	120
Triazolam	103	99	100	100	109	122
alpha-Hydroxy triazolam	135	107	92	100	113	125
4-Hydroxy triazolam	141	100	96	100	115	131

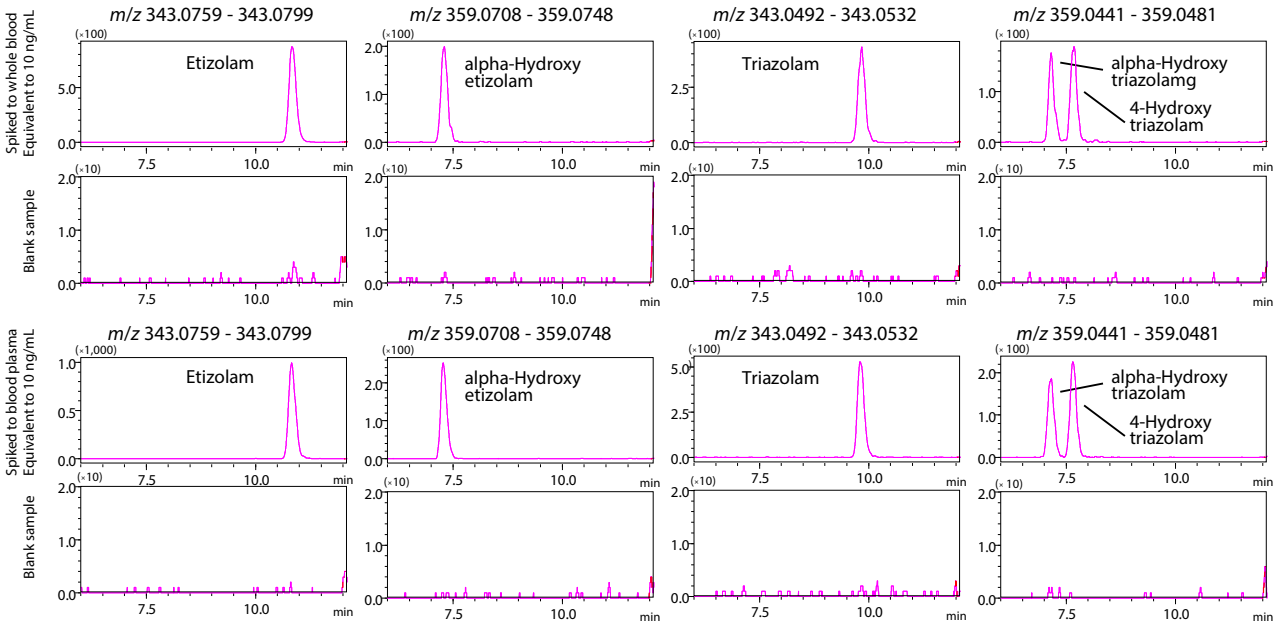

Fig. 6 Mass Chromatograms of Each Compound in Whole Blood and Blood Plasma

Table 3 Mass Errors (mDa) of Matrices and the Standard Solution at Each Concentration

Compound Name	Spike Concentration (ng/mL)					
	Standard Solution				Whole Blood	Blood Plasma
	1	5	10	100	10	10
	Absolute errors (mDa)					
Etizolam	0.237	0.152	0.088	0.064	0.271	0.310
alpha-Hydroxy etizolam	0.101	0.335	–0.229	–0.091	0.354	–0.113
Triazolam	0.233	0.142	0.060	0.308	–0.270	0.002
alpha-Hydroxy triazolam	–0.037	–0.145	–0.102	–0.290	0.314	0.048
4-Hydroxy triazolam	–0.157	0.194	0.068	0.195	0.136	0.192

Conclusion

Etizolam, Triazolam, and their metabolites were simultaneously analyzed using the LCMS-9030 high-resolution quadrupole time-of-flight mass spectrometer (Q-TOF). While only some compounds were separated using LC alone, all compounds were selectively quantified by separating and detecting alpha-Hydroxyetizolam and alpha-Hydroxytriazolam, which have a mass difference of 26 mDa, by the high resolving power of the Q-TOF.

The linearity of the calibration curves created from standard samples prepared by serial dilutions was good and the quantitative results from the whole blood and plasma samples demonstrated high accuracy.

These results of mass errors indicate that the compounds can be measured stably with high mass accuracy without being affected by concentration or matrices.

<Acknowledgments>
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- The products described in this document have not been approved or certified as a medical device under the Pharmaceutical and Medical Device Act of Japan.
- The analytical techniques described in this document cannot be used for the purpose of medical examinations.

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