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Identification of triazolam, etizolam and their metabolites in biological samples by liquid chromatography tandem mass spectrometry

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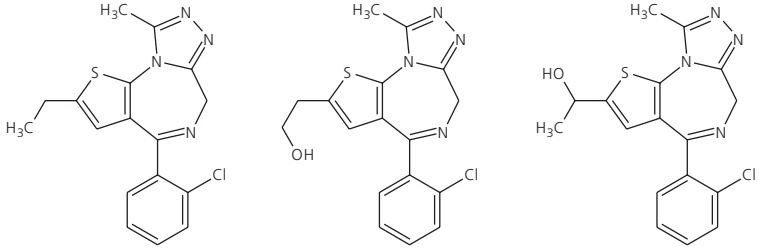
Introduction

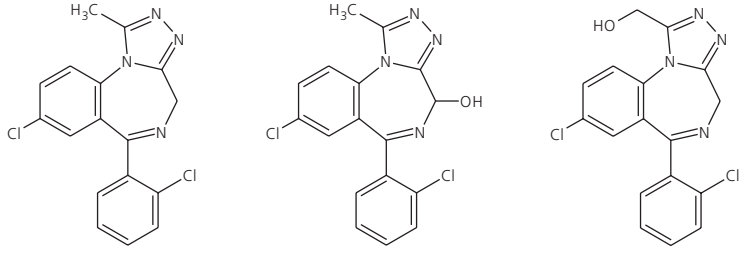
Benzodiazepines are one of the mostly widely prescribed groups of drugs because of their sedative, hypnotic, anxiolytic, antiepileptic and muscle relaxant properties. This class of compounds and their associated metabolites are also frequently present in clinical and forensic samples. For this reason, the analysis of benzodiazepines in biological fluids is of great importance to clinicians and forensic toxicologists. A key analytical challenge in the analysis of benzodiazepines is to identify etizolam, triazolam, and their metabolites (alpha-hydroxyetizolam, 8-ethylhydroxyetizolam, alpha-hydroxytriazolam and 4-Hydroxytriazolam) as a mixture, because of their very similar chemical structure, molecular weight and fragmentation during mass spectrometry. In this study we report a new high resolution separating method for the simultaneous analysis of etizolam, triazolam and their metabolites.



Etizolam
M.W. 342.07059

8-Ethylhydroxye tizolam
M.W. 358.06550

alpha-Hydroxye tizolam
M.W. 358.06550



Tria zolam
M.W. 342.04389

4-Hydroxytria zolam
M.W. 358.03881

alpha-Hydroxytria zolam
M.W. 358.03881

Fig. 1 Structure of etizolam, triazolam and their metabolites

Fig. 2 LCMS-8030 triple quadrupole mass spectrometer

Materials and Methods

Three samples were prepared: A) mixture of all standards (alpha-hydroxytriazolam, 4-hydroxytriazolam, triazolam and etizolam), B) blank metabolized matrix using human liver S9 and C) metabolised matrix of triazolam and etizolam.

In vitro metabolism of triazolam and etizolam in human liver S9:

Triazolam, etizolam, and NADPH regeneration system solution (NADP+, glucose-6-phosphate, $MgCl_2$ in H_2O), NADPH regeneration system solution (glucose-6-phosphate dehydrogenase in sodium citrate buffer), human liver S9 were mixed in 100 mM phosphate buffer (pH 7.4). The mixture was incubated at 37 deg C overnight (approx. 18 hrs). The control sample was prepared without triazolam and etizolam added. [Final concentration in incubation mixture; 40 µM triazolam and etizolam, 1.6 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM $MgCl_2$, 3.2 mg/mL S9 protein]

Extraction of incubation mixture:

The incubation mixture was extracted as follows and injected onto LC/MS/MS.

1. Add 500 µL of ice-cold acetonitrile
2. Centrifuge at 2,000 rpm for 15 min and collect the supernatant
3. Dry the supernatant in a vacuum evaporator and resolve it in 150 µL of water
4. Filter the solution through 0.2 µm syringe filter (YMC Duo-Filter)

Samples were analyzed with UHPLC and a triple quadruple mass spectrometer using following conditions.

Analytical Conditions

HPLC: Nexera UHPLC system (Shimadzu Corporation, Japan)

Column:	YMC-Triart C_{18} column, 1.9 µm, 12 nm (150 × 2 mm)
Mobile phase:	(A) 10 mM formic acid
	(B) 10 mM formic acid / acetonitrile (1/1)
Flow rate:	0.3 mL/min
Time program:	B conc. 40%(0 min)-65%(40 min)-40%(40.01-60 min)
Injection volume:	1 µL
Column temperature:	40°C

Mass spectrometer: LCMS-8030 (Shimadzu Corporation, Japan)

Ionization: Electrospray ionization, Positive
Scan type: multiple-reaction-monitoring mode (MRM)
MRM triggered automatic MS/MS data acquisition

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Results

The simultaneous analysis of drugs of abuse in clinical and forensic laboratories requires highly specific methods. The developed method in this study contained not only optimized MRM transition parameters and chromatographic conditions, but also product ion scanning which is automatically triggered once an MRM exceeds a specified threshold. The method was applied to the analysis of benzodiazepines; including etizolam, triazolam, and their known metabolites. In this experiment three samples were prepared (as described above).

Firstly, sample (A) was analyzed with the method of 12 MRM transitions, which were quantitative and qualitative transitions for etizolam, triazolam, and their known metabolites and it resulted in excellent separation for all four compounds. Next, sample (C) (blank matrix) was analyzed and no peaks were observed; therefore highlighting the excellent selectivity of the method. Sample (B) (metabolized drug) was then analyzed and three new peaks were found (in addition to the four peaks in sample (A)).

compounds	Qunatitative	CE	Qualitative	CE
Etizolam	343.05>314.10	-28	343.05>138.15	-37
Triazolam	343.05>308.20	-24	343.05>315.00	-27
8-Hydroxyetizolam (M -III)	359.05>305.05	-24	359.05>315.25	-20
alpha-Hydroxyetizolam (M-VI)	359.05>286.20	-28	359.05>287.20	-27
alpha-Hydroxytriazolam	359.05>176.20	-27	359.05>341.15	-18
4-Hydroxytriazolam	359.05>341.10	-22	359.05>111.20	-39

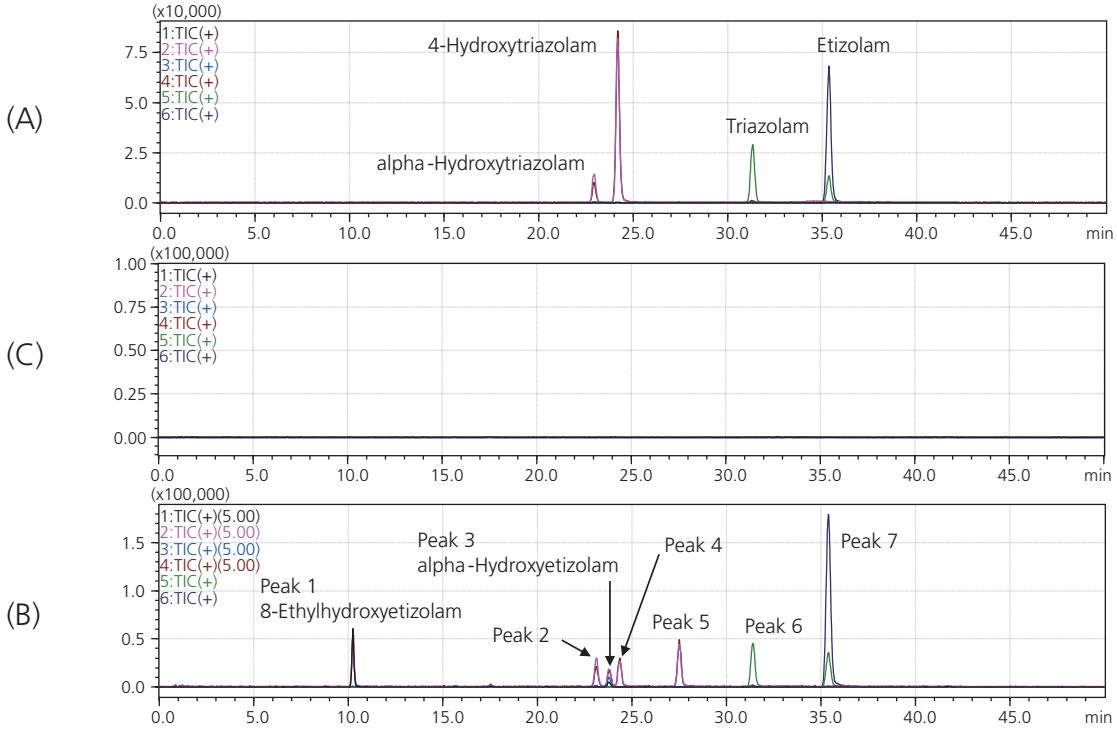

Fig. 3 12 MRM transitions for 6 drugs and metabolites (above) and MRM chromatograms for sample (A), (B), (C).

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Two of the three unknown peaks were identified as 8-Ethylhydroxyetizolam and alpha-Hydroxyetizolam as they are known metabolites. However the third unknown peak, which was detected the same MRM transition as that of metabolites of these two compounds, was not identified. Next, sample (B) was re-acquired with MRM triggered automatic MS/MS and product ion scans. These product ion scan spectra were searched against a hypnotics MS/MS library and the six previously identified peaks were assigned a high hit score. In the same manner as described here, this method is highly applicable to the screening of drugs of abuse in biological samples.

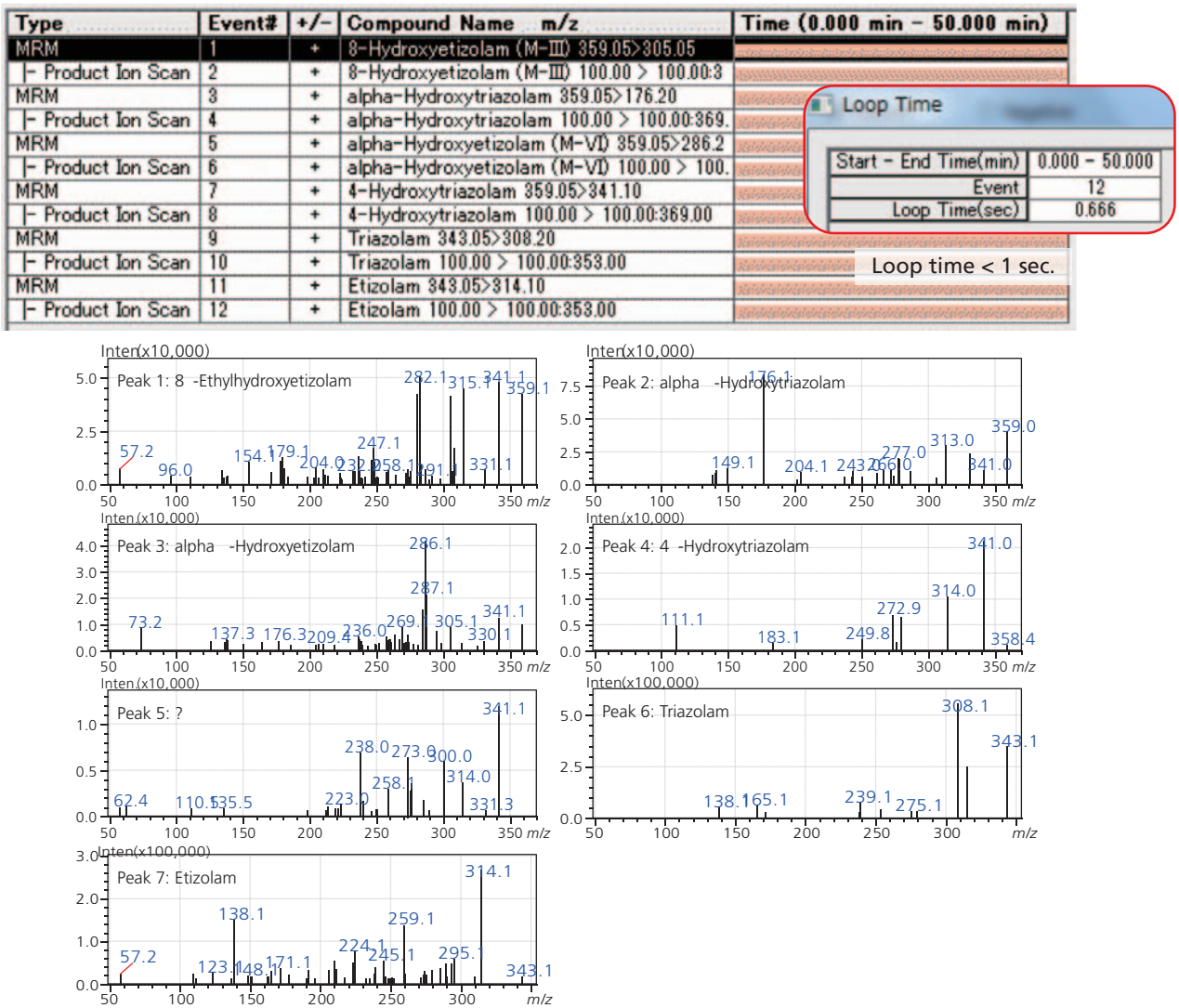

Fig. 4 MRM triggered MS/MS method and MS/MS spectra of Peak 1 to Peak 7

Conclusions

- Metabolite analysis using LC/MS/MS with small particle size column achieved high resolution separation for the simultaneous analysis of etizolam, triazolam and their metabolites.
- The metabolites were detected and confirmed with MRM triggered automatic MS/MS data acquisition.

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