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Evaluation of micro volume sample preparation technology newly designed for forensic toxicology with High Resolution Accurate Mass Spectrometry

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1. Overview

The combination of newly designed micro-volume QuEChERS extraction protocol and LC-Q-TOF-MS system to identify fragment structure using accurate MS/MS spectra.

2. Introduction

In recent years, new synthetic drugs have appeared in the illicit market. These compounds include various analogs and newly emerged compounds with different chemical structures. One of the major points of concern is the substantial ineffectiveness of current toxicological sample pretreatment and screening methods to identify the compounds in biological samples. The recent increase in the sales and abuse of substituted drugs highlighted the need for fast and reliable identification of such compounds with a simple sample preparation technology everybody can easily and smoothly implement. This work describes the identification workflow of pre-spiked drugs treated with micro-volume QuEChERS extraction protocol using high resolution accurate mass spectrometry.

Fig 1. LC-QTOF-MS system (Nexera X2 + LCMS-9030)

3. Methods and Pretreatment

Etizolam, triazolam and their metabolites were selected as the test compounds which were spiked in whole blood to confirm extraction. Acetyl fentanyl and its metabolites were selected to evaluate the performance of formula prediction software.

All samples were processed using a protocol described in Micro Volume QuEChERS kit (Shimadzu Corporation, Kyoto, Japan) which can extract drugs with a 100 µL amount of biological samples.

LC and MS conditions are shown in table 2. Two measurement modes (MS scan and MS/MS) using the quadrupole time-of-flight mass spectrometer (LCMS-9030, Shimadzu). The data processing software (LabSolutions LCMS, Shimadzu) and MS Workbook Suite (ACD/Labs, Toronto, Canada) were used for the formula prediction.

Table 1. LC and MS conditions

[LC] Nexera™ X2 System			[MS] LCMS-9030	
Analytical Column	Phenomenex Kinetex XB-C18 (2.1 mmI.D. × 100 mmL., 2.6 µm)		Ionization	: ESI (Positive)
Guard Column	Phenomenex SecurityGuard Ultra 2.1 mmI.D		Nebulizer Gas	: 3 L/min
Solvent A	10 mmol/L ammonium formate +0.1% Formic Acid – Water		Interface temperature	: 300 ℃
Solvent B	10 mmol/L ammonium formate +0.1% Formic Acid – MeOH		Desolvation Line	: 250 ℃
Gradient Program	Time (min)	%B	Heat Block temperature	: 400 ℃
	0	5	Heating Gas	: 10 L/min
	7.5	95	Drying Gas	: 10 L/min
	10	95	MS Scan Range	: m/z 100 – 1000
	10.01	5	CE Spread	:20 - 50 (V)
	15	STOP		
Flow Rate	0.3 mL/min			
Column Temp	40 ℃			

Simple sample pretreatment

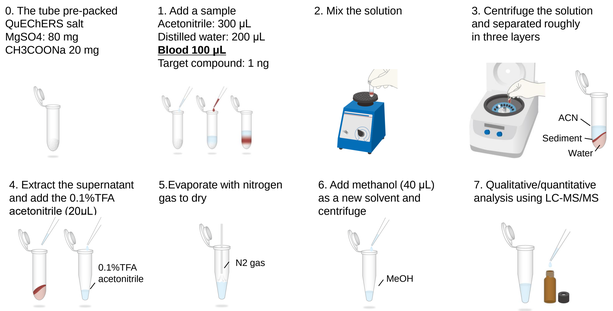


Fig 2. Sample pretreatment workflow of Micro Volume QuEChERS kit

Table 2. Target compounds

Experimental 1			Experimental 2		
Compound name	Formula	Calculated exact mass of $[M+H]^+$	Compound name	Formula	Calculated exact mass of $[M+H]^+$
Etizolam	C17H15ClN4S	343.0779	Acetyl fentanyl	C21H26N2O	323.2118
Alpha-hydroxy etizolam	C17H15ClN4OS	359.0728	Acetyl nor fentanyl	C13H18N2O	219.1492
Triazolam	C17H12Cl2N4	343.0512	Acetyl fentanyl-OH	C21H26N2O2	339.2067
Alpha-hydroxy triazolam	C17H12Cl2N4O	359.0461			
4-hydroxy triazolam	C17H12Cl2N4O	359.0461			

4. Results

Experimental 1

Fig. 3 shows an extracted-ion chromatogram (XIC) (extraction range: theoretical *m/z* value±2 mDa of each compound) obtained from a mixture of etizolam, triazolam and their metabolites (50 ng/mL). Compounds with a difference of 26 mDa, such as etizolam and triazolam or alpha-hydroxy etizolam and alpha-hydroxy triazolam, were selectively identified by the high resolving power of the Q-TOF.

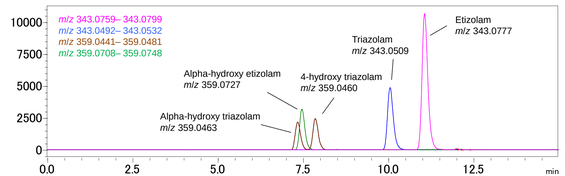


Fig 3. Mass chromatogram (extraction range: theoretical *m/z* value±2 mDa) of etizolam, triazolam, and their metabolites (50 ng/mL)

Standard solutions were diluted to 1 to 100 ppb. Spike and recovery tests were performed by adding standard to a whole blood sample (10 ppb). Quantitative results and mass accuracy are shown in Table 3. Linear calibration curves were generated for all drugs with achieved R^2 values of 0.999, and good quantitative accuracy were obtained from blood samples.

Even though an external calibration method was applied to the experiment, the mass accuracy for all tested samples was less than 0.5 mDa which include all calibration levels ranging from 1 to 100 ppb, as well as spiked samples in whole blood.

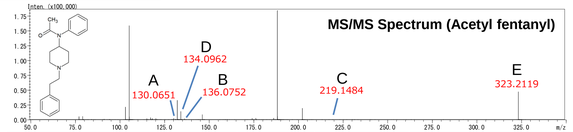
Table 3. Quantitative and mass accuracy (mDa) results of each compound from standard solution (1, 5, 10, 50, 100 ppb) and matrices (spiked 10 ppb)

Compound Name		Standard				Whole blood	
		1 ppb	5 ppb	10 ppb	100 ppb	10 ppb	R^2
Etizolam	Accuracy (%)	103	95	102	100	106	0.999
	Mass Errors (mDa)	0.24	0.15	0.09	0.06	0.27	
Alpha-hydroxy etizolam	Accuracy (%)	100	97	103	100	108	0.999
	Mass Errors (mDa)	0.10	0.34	-0.23	-0.10	0.35	
Triazolam	Accuracy (%)	100	99	101	100	107	0.999
	Mass Errors (mDa)	0.23	0.14	0.06	0.31	-0.27	
Alpha-hydroxy triazolam	Accuracy (%)	99	102	99	100	103	0.999
	Mass Errors (mDa)	-0.04	-0.15	-0.10	-0.29	0.31	
4-hydroxy triazolam	Accuracy (%)	102	96	102	100	104	0.999
	Mass Errors (mDa)	-0.16	0.19	0.07	0.20	0.14	

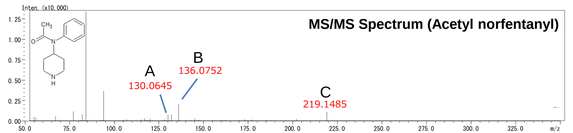
Experimental 2

Acetyl fentanyl, acetyl norfentanyl and acetyl fentanyl-OH were spiked to the whole blood and measured at each accurate MS/MS spectra to reveal their fragment structures. MS workbook Suite was used to generate theoretical fragments based on the given structure and automatically assigned with the measured MS/MS spectra. Fig. 4 shows the MS/MS spectra and assigned fragment formula of acetyl fentanyl and its metabolites. The fragmentation of these compounds by MS/MS provided three main fragment ions at *m/z* (A) 130.0651, (B) 136.0757, and (C) 219.1492, because of the same structural pattern they have. There was different fragmentation from acetyl fentanyl and acetyl fentanyl-OH. Acetyl fentanyl-OH had a unique ion at *m/z* (D) 150.0913 which was the hydroxylated ion at *m/z* (D) 134.0964 of acetyl fentanyl.

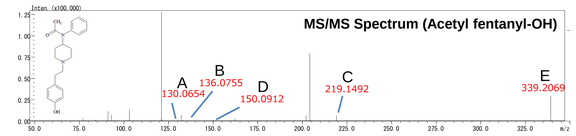
The mass accuracy for all fragment ions were less than 1 mDa with external calibration. This great mass accuracy was helpful to reduce the candidates of possible fragment patterns and enhance the efficiency of formula prediction for drugs and their metabolites.



ID	Structure	Mass errors (mDa)	ID	Structure	Mass errors (mDa)	ID	Structure	Mass errors (mDa)
A		-0.1	B		-0.5	C		-0.8
D		-0.2	E		0.1			



ID	Structure	Mass errors (mDa)	ID	Structure	Mass errors (mDa)	ID	Structure	Mass errors (mDa)
A		-0.6	B		-0.5	C		-0.7



ID	Structure	Mass errors (mDa)	ID	Structure	Mass errors (mDa)	ID	Structure	Mass errors (mDa)
A		0.3	B		-0.2	C		0.0
D		-0.1	E		0.2			

Fig 4. Accurate MS/MS spectra and automatically assigned fragments of acetyl fentanyl and metabolites. Difference was calculated from theoretical *m/z* and measured *m/z*

5. Conclusion

The combination of a newly developed sample preparation protocol with a rugged and reliable HRAM platform which has great mass accuracy helps identify unrevealed drugs.