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Rapid screening and semi-quantitative analysis for forensic drugs in blood using liquid chromatography triple quadrupole mass spectrometry

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1. Introduction

In Forensic Toxicology, LC/MS/MS has become a preferred method for the routine quantitative and qualitative analysis of drugs of abuse. LC/MS/MS allows for the simultaneous analysis of multiple compounds in a single run, thus enabling a fast and high throughput analysis. But a pretreatment is the most critical step in forensic analysis. In this study, we report a developed analytical system using a modified QuEChERS* extraction method for pretreatment in forensic analysis by high-speed triple quadrupole mass spectrometry.

*QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe): A method developed as a pretreatment method for analysis of pesticide residues in foods and agricultural products by Anastassiades et al. in 2003

2. Methods and Materials

Sample Preparation

Whole blood sample preparation was carried out by the modified QuEChERS extraction method [(1)] using Q-sep™ QuEChERS Sample Prep Packets purchased from RESTEK (Bellefonte, PA).

1) Add 0.5 mL of blood and 1 mL of distilled water into the 15 mL centrifugal tube and agitate the mixture using a vortex mixer.
2) Add two 4 mm stainless steel beads, 1.5 mL of acetonitrile and 100 µL of acetonitrile solution containing 1 ng/µL of Diazepam-d5. Then agitate using the vortex mixer.
3) Add 0.5 g of the filler of the Q-sep™ QuEChERS Extraction Salts Packet.
4) Vigorously shake the tube by hand several times, agitate well using the vortex mixer for approximately 20 seconds. Then centrifuge the tube for 10 minutes at 3000 rpm.
5) Move the supernatant to a different 15 mL centrifugal tube and add 100 µL of 0.1% TFA acetonitrile solution. Then, dry using a nitrogen-gas-spray concentration and drying unit or a similar unit.
6) Reconstitute with 200 µL of methanol using the vortex mixer. Then move it to a microtube, and centrifuge for 5 minutes at 10,000 rpm.
7) Transfer 150 µL of the supernatant to a 1.5 mL vial for HPLC provided with a small-volume insert.

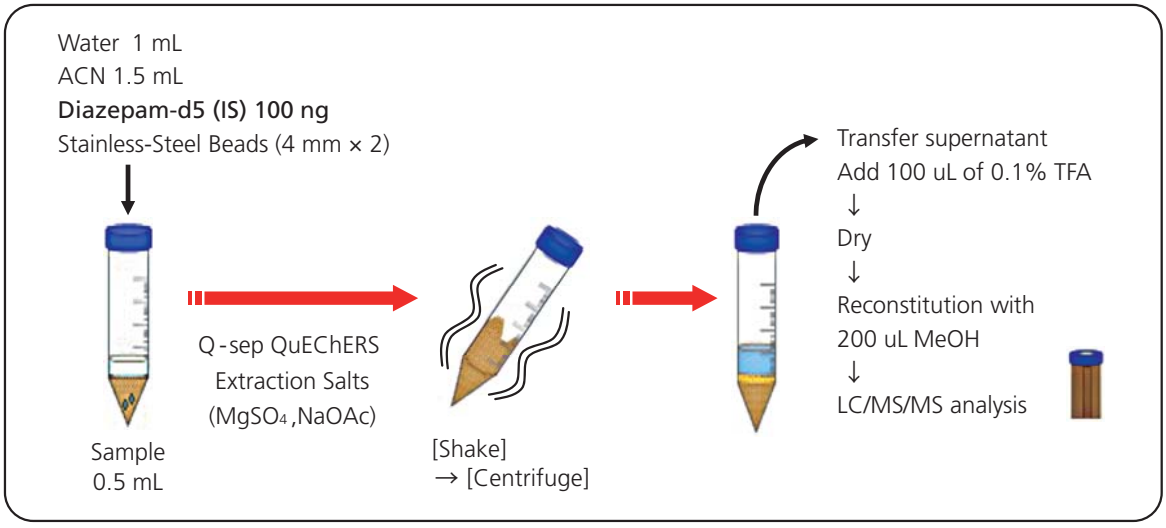

Fig. 1 Scheme of the modified QuEChERS procedure

[ref.] (1) Usui K et al, Legal Medicine 14 (2012), 286-296

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Table 1 List of analytes for forensic using LC/MS/MS

Abused Drugs [15]
Amphetamine
Benzoyl ecgonine
Cocaine
Codeine
Dihydrocodeine
Ecgonine methyl ester
Ephedrine
Ketamine
MDA
MDMA
Methamphetamine
Methylephedrine
Methylphenidate
THC
THC-COOH
Hypnotic Drugs [35]
7-Aminoflunitrazepam
7-Aminonimetazepam
7-Aminonitrazepam
8-Hydroxyetizolam (M-III)
Allylisopropylacetylurea
alpha -Hydroxybrotizolam
alpha -Hydroxytriazolam
Alprazolam
Amobarbital (neg)
Barbital (neg)
Bromazepam
Bromovalerylurea
Brotizolam
desmethyldiazepam
Diazepam
Estazolam
Ethyl loflazepate
Etizolam
Flunitrazepam
Flurazepam
Hydroxyzine
Lorazepam
Lormetazepam
Midazolam
Nimetazepam
Nitrazepam
Oxazepam
Pentobarbital (neg)
Phenobarbital (neg)
Quazepam
Temazepam
Thiamylal (neg)
Triazolam
Zolpidem
Zopiclone
Psychotropic Drugs [23]
Amitriptyline
Amoxapine
Aripiprazole
Chlorpromazine
Clomipramine
Dosulepin
Fluvoxamine
Haloperidol
Imipramine
Levomepromazine
Maprotiline
Mianserin
Nortriptyline
Olanzapine
Paroxetine
Promethazine
Quetiapine
Risperidone
Sertraline
Sulpiride
Trazodone
Trihexyphenidyl
Zotepine
Medical Drugs [27]
Acetaminophen
Acetylpheneturide
Atropine
Biperiden
Bupivacaine
Carbamazepin
Chlorpheniramine
Clonazepam
Dextromethorphan
Diclofenac
Diltiazem
Diphenhydramine
Diprophyline
Ethenzamide
Glibenclamide
Glimepiride
Lidocaine
Loxoprofen (neg)
Mepivacaine
Mexiletine
Mirtazapine
Pancuronium
Pentazocine
Salicylic acid (neg)
sildenafil
Vecuronium
Warfarin
Natural Toxines [2]
Aconitine
Colchicine
Pesticides [4]
Diquat
Fenitrothion(MEP)
Malathion
Methomyl

LC-MS/MS Analysis

Treated samples were analyzed using a Nexera UHPLC system coupled to a LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corporation, Japan) with LC/MS/MS Rapid Tox. Screening Database. The Database contains product ion scan spectra for 106 forensic and toxicology-related compounds of Abused drugs, Psychotropic drugs and Hypnotic drugs etc (Table 1) and provides Synchronized Survey Scan® parameters (product ion spectral data acquisition parameters based on the MRM intensity as threshold) optimized for screening analysis. Samples were separated on a Shim-pack FC-ODS column. A flow rate of 0.3 mL/min was used together with a gradient elution.

Analytical Conditions

HPLC (Nexera UHPLC system)

Column	: Shim-pack FC-ODS (2.0 mmI.D. x 150 mmL., 3 um)
Mobile Phase A	: 10 mM Ammonium formate
Mobile Phase B	: Methanol
Gradient Program	: 5%B (0 min) - 95%B (15-20 min) - 5%B (20.01 - 30 min)
Flow Rate	: 0.3 mL / min
Column Temperature	: 40°C
Injection Volume	: 5 uL

Mass (LCMS-8040 triple quadrupole mass spectrometry)

Ionization	: ESI
Polarity	: Positive & Negative
Probe Voltage	: +4.5 kV (ESI-Positive mode); -3.5 kV (ESI-Negative mode)
Nebulizing Gas Flow	: 1.5 L / min
Drying Gas Pressure	: 10 L / min
DL Temperature	: 250°C
BH Temperature	: 400°C

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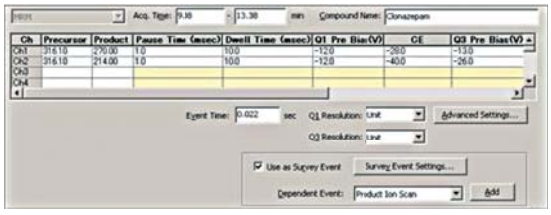
Using a polarity switching speed of 15 msec and a scan speed of 15,000 u/sec, product ion spectra were generated in both positive and negative ionization which could be matched against a user library of 106 compounds an automated aid to screening and compound identification. Fast polarity switching helps to provide information rich product ion spectra resulting in better detection and identification.

Type	Event#	+/−	Compound Name m/z	Time (0.620 min – 19.080 min)
MRM	69	+	Clonazepam 316.10>270.00, 316.10>214.00	
├ Product Ion Scan	70	+	Clonazepam 100.00 > 20.00:326.00	
MRM	71	+	Nitrazepam 282.10>236.05, 282.10>180.15	Positive
├ Product Ion Scan	72	+	Nitrazepam 100.00 > 20.00:292.00	
MRM	209	−	Amobarbital (neg) 225.15>42.00, 225.15>182.00	
├ Product Ion Scan	210	−	Amobarbital (neg) 100.00 > 20.00:235.00	
MRM	211	−	Pentobarbital (neg) 225.15>42.10, 225.15>182.10	Negative
├ Product Ion Scan	212	−	Pentobarbital (neg) 100.00 > 20.00:235.00	
MRM	73	+	Alpha-Hydroxytriazolam 359.05>176.20, 359.05>341.15	
├ Product Ion Scan	74	+	Alpha-Hydroxytriazolam 100.00 > 20.00:369.00	

MRM parameter



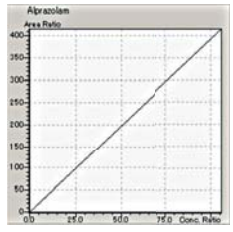
Product Ion Scan parameter

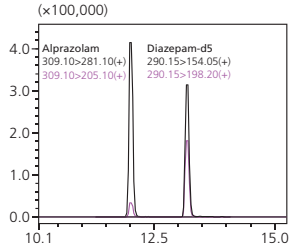
Fig. 2 User Interface of MRM-Product Ion Scan setting at LabSolutions software

Simple Quantitative Method

Based on the chromatogram obtained by injection of a fixed volume of individual reference standard solutions, calculate the ratio of peak area of the reference standard to that of the internal standard (Diazepam-d5), and prepare a calibration curve by plotting these ratios on the ordinate against the ratio of the amount of the reference standard to that of the internal standard on the abscissa.
1st coefficient and intersection were calculated from the alibration curve and we registered them with the LCMS method (Fig. 3).

ID#	Name	m/z	Ref. Ions	Ret. Time	1st Coefficient	Intersection
1	0_Diazepam-d5	290.15>154.05	290.15>198.20	13.235	0.000000e+000	0.000000e+000
2	7-Aminoflunitrazepam	284.10>135.10	284.10>226.15	8.716	1.150000e+000	4.210000e-002
3	7-Aminonimetazepam	266.10>135.05	266.10>209.10	9.081	4.030000e+000	2.370000e-001
4	7-Aminonitrazepam	252.10>121.05	252.10>94.10	7.877	2.240000e+000	9.730000e-002
5	8-Hydroxyetizolam (M-III)	359.05>315.25	359.05>305.05	10.047	2.630000e-001	1.950000e-002
6	Acetaminophen	152.00>110.00	152.00>65.05	4.367	3.960000e+000	2.330000e-001
7	Acetylpheneturide	249.10>119.05	249.10>91.05	11.975	5.090000e+000	2.010000e-001
8	Aconitine	646.00>586.30	646.00>104.95	12.216	1.500000e-002	4.160000e-003
9	Allylisopropylacetylurea	185.00>55.05	185.00>97.10	10.716	5.760000e-001	-2.640000e-003
10	Alpha-Hydroxybromazolam	409.00>336.85	409.00>284.30	11.544	1.240000e-002	2.840000e-004
11	Alpha-Hydroxytriazolam	359.05>176.20	359.05>341.15	11.424	2.220000e-002	6.810000e-004
12	Alprazolam	309.10>281.10	309.10>205.10	12.102	3.930000e+000	1.360000e-002
13	Amitriptyline	278.20>91.05	278.20>105.05	14.467	6.310000e+000	8.280000e-002
14	Amobarbital (neg)	225.15>42.00	225.15>182.00	11.404	2.840000e-002	2.970000e-003
15	Amoxapine	314.10>270.95	314.10>70.05	13.555	3.180000e+000	2.040000e-001





ID#	Name	Ret. Tim	Area	Conc.	Unit
1	0_Diazepam-d5	13.158	1715388	0.200	ng/ul
12	Alprazolam	12.006	2415926	0.071	ng/ul

Fig. 3 Registration of 1st coefficient and intersection by calibration curve

3. Results and Discussion

Determination of drugs in whole blood is often necessary in forensic analysis because of the difficulty in obtaining serum or plasma. Conventional procedures to analyze drugs in complex matrices like whole blood involve tedious, time consuming, expensive, and complex steps, and possible sample loss and contamination problems are not unusual. QuEChERS is a sample preparation technique that was developed for the extraction of multi-class pesticide residue from fruits and vegetables, in 2003.

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In this study, we report a modified QuEChERS extraction method for pretreatment in forensic analysis and a simple quantitative method including information of calibration curve of each compound by high-speed triple quadrupole mass spectrometry. To achieve a highly specific and sensitive detection in screening and quantitation a MRM triggered product ion scanning method using a fast polarity switching speed and a fast scan speed was applied to 108 components including illicit drugs, psychotropics, hypnotics, pesticides and other substances. As the MRM acquisition time was very fast, this enabled product ion spectra to be generated in both positive and negative ionization mode which could be matched against a user library of compounds as an automated aid to screening and compound identification.
The recovery of 24 compounds from whole blood are summarized in Table 2. The range is from 57 to 98%.

Table 2 Recovery test of 24 compounds

	Compounds	Recovery (%)
(IS)	Diazepam-d5	84.85
Abused Drugs	Dihydrocodeine	57.72
	Methamphetamine	70.15
	Methylephedrine	63.78
Hypnotic Drugs	7- Aminoflunitrazepam	91.23
	7- Aminonitrazepam	95.29
	Bromovalerylurea	82.71
	Flunitrazepam	87.52
	Phenobarbital (neg)	64.68
	Quazepam	91.05
	Zolpidem	96.91
Psychotropic Drugs	Amoxapine	82.04
	Chlorpromazine	68.30
	Clomipramine	93.16
	Fluvoxamine	92.84
	Levomepromazine	71.17
	Olanzapine	61.62
	Paroxetine	90.49
	Quetiapine	91.68
	Sertraline	97.97
	Zotepine	95.20
Medical Drugs	Acetaminophen	93.84
	Chlorpheniramine	83.37
	Diclofenac	71.36
	Mirtazapine	86.88
	Average	82.63

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The results of instrument difference using standard (each 0.05 ng/µL of 16 compounds) among 3 universities are summarized in Table 3.

Table 3 Instrument difference Test of 16 compounds

	X University [LCMS -8030]			Y University [LCMS-8040]			Z University [LCMS-8030plus]		
Compounds	r.t	Area	Conc. (ng/u)l	r.t	Area	Conc. (ng/u)l	r.t	Area	Conc. (ng/u)l
Diazepam-d5	13.01	541837	0.500	13.04	510849	0.500	13.16	1285894	0.200
Alprazolam	11.89	152300	0.035	11.89	252411	0.057	12.04	2706623	0.106
Aripiprazole	15.38	298571	0.042	15.51	353307	0.048	15.89	2539919	0.058
Atropine	6.84	517258	0.096	6.92	742471	0.135	6.69	1439814	0.045
Brotizolam	12.04	133731	0.092	12.04	110326	0.073	12.16	697512	0.079
Estazolam	11.49	241206	0.058	11.52	309021	0.071	11.65	1855497	0.073
Ethyl loflazepate	13.07	258218	0.065	13.10	162485	0.040	13.22	1777912	0.073
Etizolam	12.10	206778	0.067	12.13	120773	0.037	12.25	1159548	0.063
Flunitrazepam	11.27	139191	0.064	11.28	87325	0.039	11.43	770711	0.057
Haloperidol	11.44	946233	0.049	11.63	1612548	0.080	11.52	6093979	0.052
Lidocaine	11.50	8382533	0.060	12.10	1837944	0.013	12.16	92627857	0.111
Nimetazepam	11.49	199451	0.127	11.49	173364	0.107	11.63	984083	0.105
Risperidone	11.07	1376790	0.047	11.50	840337	0.028	12.25	8761806	0.049
THC	16.77	11281	0.052	16.80	126172	0.557	17.01	42336	0.032
THC-COOH	14.76	26939	0.051	15.10	13905	0.025	14.95	399524	0.126
Triazolam	11.78	88902	0.068	11.77	155441	0.114	11.91	931014	0.115

4. Conclusions

- The validated sample preparation protocol can get adequate recoveries in quantitative works for all compounds ranging from acidic to basic.
- The combination of the modified QuEChERS extraction method and high-speed triple quadrupole LC/MS/MS with a simple quantitative method enable to acquire reliable data easily.

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