Int J Clin Exp Med 2019;12(11):13135-13146
www.ijcem.com /ISSN:1940-5901/IJCEM0097481

Original Article

Simultaneous determination of benzodiazepines, Z-drugs and metabolite concentrations in human whole blood by UPLC-MS/MS

Duoqi Xu[1,2], Lirui Sun[3], Jifen Wang[4], Yanjun Zhao[5], Leiping Zhang[6]

[1]Guangdong Provincial Key Laboratory of Emergency Test for Dangerous Chemicals, Guangzhou Province, China; [2]China University of Political Science and Law, Beijing City, China; [3]Department of Pharmacy, The First Hospital, Jilin University, Changchun, Jilin Province, China; [4]School of Criminal Science and Technology, People's Public Security University of China, Beijing City, China; [5]Department of Pharmacy, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang Province, China; [6]Institute of Forensic Science, Ministry of Public Security, Beijing City, China

Received May 24, 2019; Accepted September 5, 2019; Epub November 15, 2019; Published November 30, 2019

Abstract: Objective: To establish a simple, easy and quick method to screen benzodiazepines, Z-drugs, and their metabolites in whole blood samples by ultra-performance liquid chromatography tandem electrospray ionization triple quadrapole-mass spectrometry (UPLC-TQ/MS). Methods: Samples were prepared by modified Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method. After being extracted with 0.1% acetic acid and dehydrated by anhydrous magnesium sulfate, the targets were separated and analyzed on a ZORBAX Eclipse PlusC18 (2.1 mm*100 mm, 1.8 μm) column by gradient elution with 0.1% formic acid-0.1% formic acid/acetonitrile as mobile phase, then ionized with positive electrospray, and finally detected under a multiple reaction monitoring mode. Results: Chromatographic separation was achieved in less than 5 min. Limits of detection (LODs) varied from 0.5 to 2 ng/mL. Conclusion: The experimental method outlined in this paper showed high accuracy, linearity, and repeatability, which can meet the demand of the analysis in actual cases.

Keywords: QuEChERS, benzodiazepines, Z-drugs, UPLC-MS/MS

Introduction

Benzodiazepines and Z-drugs are the most commonly prescribed hypnotic agents with definite curative effects and low toxicity, and are widely applied for insomnia therapy [1, 2]. However, they are also used by nefarious individuals with ill intent [3, 4]. Benzodiazepines and Z-drugs have also been detected in some cases of mixed drug abuse [5]. Therefore, quick and accurate detection of benzodiazepines and Z-drugs in human whole blood is a hotspot in Laboratory Science.

In the toxicological analysis of bio-samples, the most critical step is the pretreatment of human samples. The method of Solid Phase Extraction (SPE) requires a variety of different conditions for the extraction of different classes of compounds; in addition to being more expensive and relying on a manifold vacuum, which is not always available in a laboratory setting [6, 7]. The method of Liquid-liquid Extraction (LLE) consumes too much organic reagents and is prone to emulsification during the experiment [8]. Therefore, it is necessary to establish more effective pretreatment methods.

The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method, is an emerging sample pretreatment technique which is characterized by its speed, low cost, high effectiveness, ruggedness and safety; and it is becoming increasingly popular in a number of fields, including the food, clinical, veterinary and forensic science industries [9-12]. Briefly, the QuEChERS method required two steps: first, extraction-partitioning; second, dispersive solid-phase extraction (DSPE) clean-up and concentration acquisition [13, 14]. With an expanded application of QuEChERS, laboratory personnel can further optimize the sample sol-

vent for extraction, the sample purification process, and the concentration process in order to reduce potential matrix effects [15-17].

Currently, the detection and analysis methods of benzodiazepines reported in the literature mainly include two kinds of methods: one includes ultraviolet spectrophotometry, fluorescence spectrophotometry, electrochemical analysis, thin-layer scanning optical density method, immunoassay; and another includes high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry (LC-MS) [18]. The former requires higher purity of the sample, but its effectiveness of quantitative identification is lower. The latter is used more often in practice. However, HPLC cannot detect drugs with very low concentration or differentiate between two drugs with similar retention times. GS-MS may impact the drug during the analysis because of the hot environment. LC-MS is the main way to study benzodiazepines and Z-drugs in bio-samples.

In the present study, we developed a simplified extraction method for blood samples with the aim of developing an easy and quick method to extract various analytes like benzodiazepines, Z-drugs, and their metabolites from blood specimens and analyzed them via UPLC-MS/MS, in hope to apply this screening method to real specimens.

Materials and methods

Chemicals and reagents

The standard drugs we used were obtained as follows: acetonitrile (analytical grade) and formic acid (analytical grade) were purchased from Aladdin biochemical technology co., LTD, Shanghai, China; alprazolam, a-hydroxyalprazolam, clonazepam, 7-aminoclonazepam, temazepam, zolpidem, zolpidem 6-carboxylic acid (ZCA), and zolpidem phenyl-4-carboxylic acid (ZPCA) were obtained from Sigma-Aldrich (St. Louis, MO, USA); chlordiazepoxide, diazepam, nordazepam, estazolam, flunitrazepam, 7-aminoflunitrazepam, midazolam, a-hydroxymidazdam, nitrazepam, 7-aminonitrazepam, chlorpromazine, triazolam, and a-hydroxytriazolam were obtained from Wako Pure Chemical Industries (Osaka, Japan); zopiclone, zopiclonen-oxide, n-desmethylzopiclone were purchased from Tocris Bioscience (Bristol, UK); zaleplon, 5-oxozaleplon and desethylzaleplon were purchased from Toronto Research Chemicals (Canada); melatonin and 6-hydroxymelatonin were purchased from Cerilliant (Round Rock, TX, USA).

Standard stock solutions of the 29 above-mentioned drugs were prepared in acetonitrile and stored in the dark at -10°C. A mixture containing the standard stock solutions (1 μg/mL) of all analytes was prepared in acetonitrile; the working standard solutions were prepared and diluted into 0.1 ng/mL to 100 ng/mL by acetonitrile.

Sample preparation

A sample of 200 μL mixed standards was added to the whole blood samples (500 μL, the samples came from the healthy volunteers). A sample of 300 mg anhydrous magnesium sulfate ($MgSO_4$) was then added into the test tube, and immediately vortex-mixed for 10 s. Further, a volume of 1.3 mL acetonitrile containing 0.1% formic acid was added into the tube containing the mixture of $MgSO_4$. Finally, the above mixture was separated by centrifugation (8,000 r/min, 10 min) after being shaken for 5 min. The extracting solution was transferred to an autosampler vial after the filtration with 0.22 μm organic membrane.

UPLC-MS/MS analysis

The LC-MS/MS system consisted of a QTRAP® 5500 triple quadruple linear ion trap mass spectrometer (AB Sciex, USA) fitted with a Shimadzu 30A Ultra Performance LC (Shimadzu, Japan). The Waters ACQUITY UPLC®BEH C18 column (2.1 mm*150 mm, 1.7 μm) and ZORBAX Eclipse PlusC18 column (2.1 mm*150 mm, 1.8 μm) column were used, respectively. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Temperature was set to 40°C for the column oven. The gradient was programmed as follows: 0~0.3 min, 10% (B); 0.3~3 min, 10%~90% (B); 3~3.5 min, 90% (B); 3.5~3.51 min, 90%~10% (B); 3.51~4.5 min, 10% (B). The total run time was 4.5 min with a flow rate of 0.4 mL/min. The injection volume was 2 μL.

The mass spectrometer was operated in the electrospray ionization (ESI) positive ion mode.

Parameters for the multiple reaction monitoring (MRM) method were optimized by direct infusion of analytes.

Validation study

The analytical performance of the above method was examined by looking at its linearity, recovery, matrix effects, limit of detection (LOD), and limit of quantification (LOQ). Standard curve linearity was measured by the ratio of the analyte peak area to the IS area versus nominal concentration of standards by weighted linear regression ($1/X^2$). The analyte concentration at which the signal-to-noise ratio (S/N) was greater than 3 was chosen for the LOD and that with less than 20% coefficient of variation (CV) for precision and less than ±20% for bias was selected for the LOQ. The recovery and matrix effect were evaluated for three different concentrations. Recovery of the extraction procedure (RE, %) was evaluated by comparing the signal obtained when the analytes were added to a blank sample before (n=3) to the signal obtained when the same amounts of analytes were added after extraction (n=3) at three concentrations, as proposed by Matuszewski [19].

Results

Optimization of the chromatographic conditions

This study compared the Waters ACQUITY UPLC®BEH C18 column (2.1 mm*150 mm, 1.7 μm) and ZORBAX Eclipse PlusC18 column (2.1 mm*150 mm, 1.8 μm). Our findings indicated that the latter provided better separation results with a better peak shape (data not shown). Therefore, the ZORBAX Eclipse PlusC18 column (2.1 mm*100 mm, 1.8 μm) was used.

Our optimization experiments demonstrated that when using water (A) and acetonitrile (B) for the mobile phase, the mixed targets have close retention times and cannot be properly separated. However, when using 0.1% formic acid in water (V/V) (A) and 0.1% formic acid-acetonitrile (V/V) (B) for the mobile phase, the mixed targets achieved ideal separation (see **Figure 1**).

Optimization of MS conditions

The optimal conditions were as follows: ionspray voltage was 5,500 V, source temperature was 600°C, curtain gas flow was 30psi, the collisionally activated dissociation (CAD) gas setting was high, the nebulizer gas (GS1) pressure was 55psi, and the heating gas (GS2) pressure was 50psi. **Table 1** presents the optimal conditions of drugs and metabolites.

Linearity and limit of detection (LOD) and limit of quantification (LOQ)

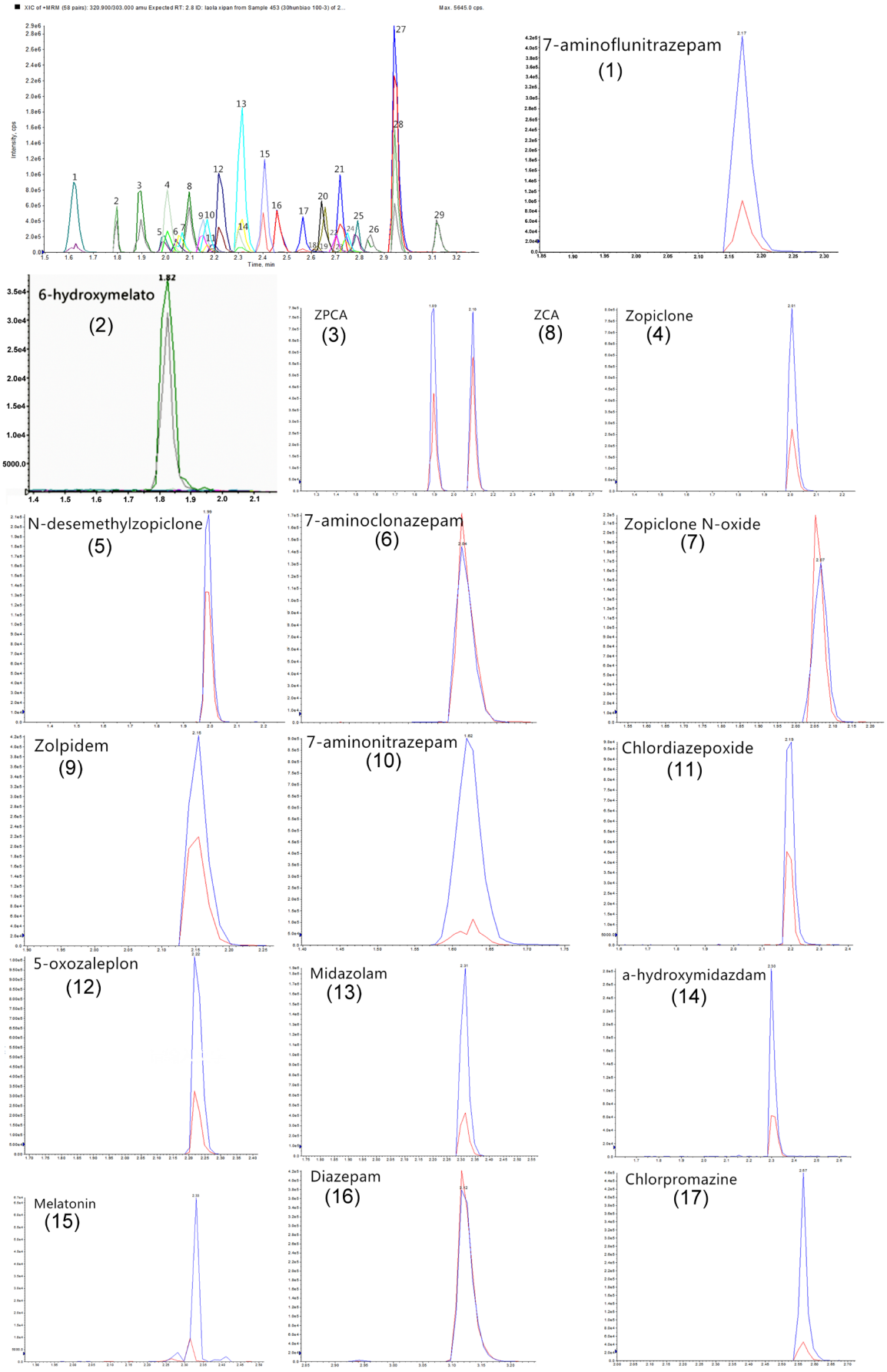
The mean values of correlation coefficients (r) and the determined values of LOD are shown in **Table 2**. The concentration of each compound showed good linearity (r>0.997). Limits of detection (LODs) varied from 0.5 to 2 ng/mL.

Recovery and matrix effects

Table 3 summarizes the recoveries, and matrix effects of each analyte from the blood samples. The results showed that QuEChERS pretreatment combined with UPLC-MS/MS had the advantages of being quick and simple, with high sensitivity, a stable matrix effect and high recovery in the analysis of benzodiazepines and Z-drugs.

Discussion

Matrix effect refers to the phenomenon that occurs when substances (other than the analyte) directly or indirectly affect analyte response during the sample measurement process [20]. With an expanded application of QuEChERS, according to our specific experimental conditions, laboratory personnel can further optimize the sample solvent for extraction [15], the sample purification process [16], and the concentration process in order to reduce potential matrix effects [17]. A blood sample's matrix composition is extremely complex, primarily made up of endogenous phospholipids, fat, cholesterol, triglycerides, and proteins [21, 22]. To prevent the matrix effects from affecting quantitative detection due to complex matrix components in LLC detection, organic solvent extraction was conducted. According to the reviewed literature [22], formic acid can precipitate protein and fat. From the point of view of selectivity, sensitivity, and separation efficiency, acetonitrile is more desirable for the mobile phase than methanol. The addition of an appropriate volume of formic acid has the potential to increase ionization efficiency. Previous studies have found that adding an appropriate amount of formic acid in an organic solvent (0.1%, V/V) could increase protein pre-


XIC of +MRM (58 pairs): 320.900/303.000 amu Expected RT: 2.8 ID: laola xipan from Sample 453 (30hunbiao 100-3) of 2...
Max. 5645.0 cps.
Intensity, cps
Time, min
7-aminoflunitrazepam
(1)
6-hydroxymelato
(2)
ZPCA
(3)
ZCA
(8)
Zopiclone
(4)
N-desemethylzopiclone
(5)
7-aminoclonazepam
(6)
Zopiclone N-oxide
(7)
Zolpidem
(9)
7-aminonitrazepam
(10)
Chlordiazepoxide
(11)
5-oxozaleplon
(12)
Midazolam
(13)
a-hydroxymidazdam
(14)
Melatonin
(15)
Diazepam
(16)
Chlorpromazine
(17)

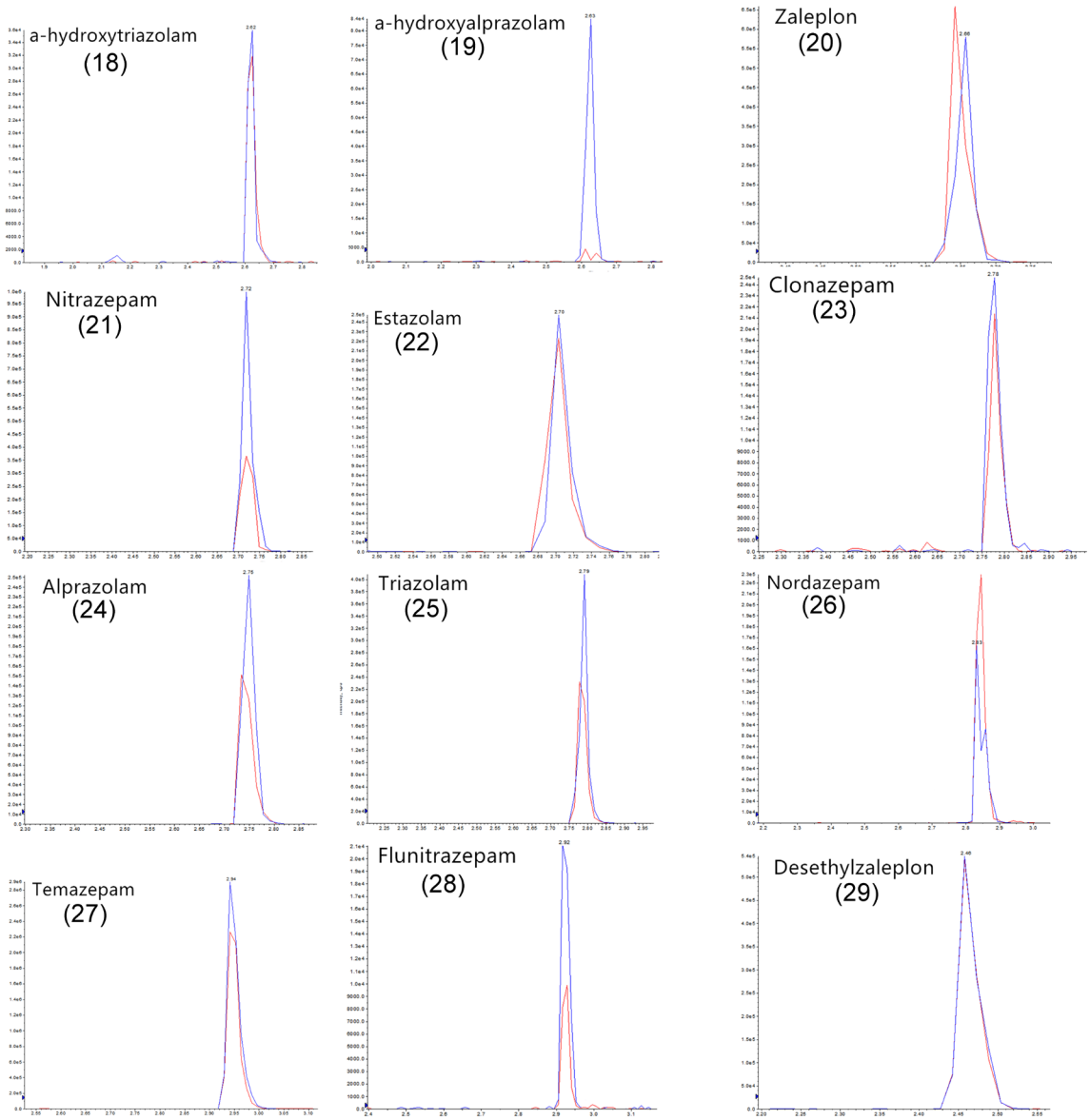


Figure 1. Total ion chromatogram of the mixed targets. 1, 7-aminoflunitrazepam; 2, 6-hydroxymelatonin; 3, zolpidem phenyl-4-carboxylic acid (ZPCA); 4, zopiclone; 5, N-desmethylzopiclone; 6, 7-aminoclonazepam; 7, zopiclone N-oxide; 8, zolpidem 6-carboxylic acid (ZCA); 9, zolpidem; 10, 7-aminonitrazepam; 11, chlordiazepoxide; 12, 5-oxo-zaleplon; 13, midazolam; 14, a-hydroxymidazdam; 15, melatonin; 16, desethylzaleplon; 17, chlorpromazine; 18, a-hydroxytriazolam; 19, a-hydroxyalprazolam; 20, zaleplon; 21, nitrazepam; 22, estazolam; 23, clonazepam; 24, alprazolam; 25, triazolam; 26, nordazepam; 27, temazepam; 28, flunitrazepam; 29, diazepam.

cipitation [23]. Therefore, in our study, 0.1% formic acid-acetonitrile (V/V) was used as the post-optimization extraction solvent.

To avoid matrix effects caused by buffering salt in MS, no buffer solution was used in the extraction process. The anhydrous magnesium sulfate was used as a dehydrating salt in our experiment. The magnesium sulfate we added to the blood was quickly wrapped by blood during shaking, and formed tiny blood-magnesium sulfate particles. These particles effectively increased the contact area of the target drug and the extraction solvent. Consistent with the relevant research reports, our results revealed that sodium sulfate easily compacted with water and had difficulty properly mixing with the blood [24].

The purpose of sample purification is to remove impurities and keep the targets as much as possible, so as to ensure a high recovery rate

Table 1. Drugs, Q1 and Q3 mass, retention time (Rt; min), declustering potential and collision energy

Compounds	Q1 mass	Q3 mass	Rt (min)	DP (V)	CE (eV)	Lonization mode
Zaleplon	306.1	236.0*	2.66	140	36	Positive
		264.4			28	
5-oxozaleplon	322.2	280.2*	2.22	140	22	Positive
		252.2			35	
Desethylzaleplon	278.2	236.4*	2.46	90	32	Positive
		260.3			26	
Zolpidem	308.1	263	2.15	100	46	Positive
		235*			34	
ZCA	338.2	265*	2.10	130	47	Positive
		293			36	
ZPCA	338.2	265*	1.89	130	47	Positive
		293			36	
Zopiclone	389	245*	2.01	70	22	Positive
		345			12	
Zopiclone N-oxide	405.1	143.1*	2.07	90	14	Positive
		245.2			19	
N-desmethylzopiclone	375.1	245*	1.99	60	24	Positive
		331.1			14	
Melatonin	233.1	174*	2.36	70	18	Positive
		159			35	
6-hydroxymelatonin	249.1	190.1*	1.80	70	19	Positive
		158			33	
Alprazolam	309	280.9*	2.75	130	33	Positive
		205			58	
A-hydroxyalprazolam	325	297.1*	2.63	200	38	Positive
		278.8			34	
Triazolam	342.9	307.9*	2.79	130	39	Positive
		314.9			39	
A-hydroxytriazolam	359	331*	2.61	140	38	Positive
		175.9			38	
Midazolam	326	290.9*	2.31	170	38	Positive
		243.9			35	
A-hydroxymidazdam	342.1	324	2.30	150	29	Positive
		203.1*			39	
Clonazepam	316.1	214*	2.78	130	51	Positive
		240.9			47	
7-aminoclonazepam	285.9	222*	2.04	160	34	Positive
		121			42	
Diazepam	285.1	193.2	3.12	150	37	Positive
		154.0*			43	
Nordazepam	271.1	243*	2.83	230	31	Positive
		165			46	
Flunitrazepam	314	268.1*	2.94	160	35	Positive
		239			46	
7-aminoflunitrazepam	284	135*	2.17	180	34	Positive
		225.9			36	
Nitrazepam	281.9	236*	2.72	160	32	Positive
		207.1			47	

7-aminonitrazepam	252.2	120.9*	1.62	170	35	Positive
		224.2			26	
Temazepam	301	254.7*	2.94	130	30	Positive
		283			20	
Estazolam	295.1	266.9*	2.70	130	31	Positive
		205			53	
Chlordiazepoxide	327.1	270*	2.19	130	29	Positive
		192.1			56	
Chlorpromazine	319.4	86*	2.78	150	31	Positive
		245.9			31	

Note: ZCA, zolpidem 6-carboxylic acid; ZPCA, zolpidem phenyl-4-carboxylic acid; *Qualitative ion pair.

Table 2. Calibration range, correlation coefficients, LODs and LOQs values of devised method (n=9)

Compounds	Calibration range (ng/mL)	Correlation coefficient (r)	LOD (ng/mL)	LOQ (ng/mL)
Zaleplon	1-100	0.9932	0.50	1.00
5-oxozaleplon	1-100	0.9971	0.50	1.00
Desethylzaleplon	1-100	0.9958	0.50	1.00
Zolpidem	1-100	0.9986	0.50	1.00
Zca	1-100	0.9961	0.50	1.00
Zpca	1-100	0.9979	0.50	1.00
Zopiclone	2-100	0.9923	1.00	2.00
Zopiclone N-oxide	2-100	0.9951	1	2.00
N-desmethylzopiclone	1-100	0.9999	0.50	1.00
Melatonin	1-100	0.9988	0.50	1.00
6-hydroxymelatonin	1-100	0.9971	0.50	1.00
Alprazolam	2-100	0.9998	1.00	2.00
A-hydroxyalprazolam	5-100	0.9997	1.00	5.00
Chlordiazepoxide	2-100	0.9900	1.00	2.00
Chlorpromazine	1-100	0.9980	0.50	1.00
Triazolam	1-100	0.9992	0.50	1.00
A-hydroxytriazolam	5-100	0.9999	2.00	5.00
Midazolam	1-100	0.9931	0.50	1.00
A-hydroxymidazdam	2-100	0.9990	1.00	2.00
Clonazepam	5-100	0.9932	2.00	5.00
7-aminoclonazepam	2-100	0.9949	1.00	2.00
Diazepam	1-100	0.9949	0.50	1.00
Nordazepam	2-100	0.9958	1.00	2.00
Flunitrazepam	1-100	0.9938	0.50	1.00
7-aminoflunitrazepam	1-100	0.9982	0.50	1.00
Nitrazepam	1-100	0.9998	0.50	1.00
7-aminonitrazepam	1-100	0.9996	0.50	1.00
Temazepam	1-100	0.9972	0.50	1.00
Estazolam	2-100	0.9980	1.00	2.00

while reducing matrix effects. In this study we investigated three purification methods. The first is the conventional QuEChERS method (Method 1): after centrifugation, the extract went through the ethylenediamine-N-propyl (PSA) solid phase extraction column and was eluted with 0.1% formic acid-acetonitrile elution. PSA was used to remove organic acids, phenols, and a small amount of pigments, hence effectively eliminating any interference from organic acids and pigments in the pesticide's residue determination, thereby reducing matrix effects. The second method applied ISOLUTE® PLD phospholipid removal plate (Method 2): after centrifugation, the extract was purified with a 96-well ISOLUTE® PLD phospholipid removal plate, which can effectively remove phospholipids from the blood to decrease the influence of matrix effects on the experiment. The third method skipped the purification process altogether (Method 3): after centrifugation, the extract was directly filtered through a 0.22 μm organic membrane, and the filtrate was subjected to LC/MS. Our results showed that the first two methods effectively reduced matrix effects, however, the detection limit was high, especially for the metabolites of α-Hydroxyalprazolam, Hydroxymidazolam, and Z-line drugs (data not shown). Method 3 provided a higher recovery rate while completing extraction and purification in one step, which saves time and reduces costs as well. Given these results, we used the third method for this experiment.

Table 3. RE, ME and RSD of analysis of test drugs at three different concentrations (n=3)

Compounds	Spiked concentration (ng/mL)	Recovery (%)	Matrix effect (%)	Precision (RED %)	
				Intraday	Interday
Zaleplon	1	84.48	99.8	6.74	12.25
	10	94.9	97.06	8.47	10.12
	100	90.1	96.6	5.69	6.77
5-oxozaleplon	1	84.61	96.20	2.29	14.08
	10	90.90	94.33	5.71	11.28
	100	113.1	116.5	6.56	7.40
Desethylzaleplon	1	106.3	128.2	7.65	13.84
	10	85.04	98.49	1.54	14.96
	100	89.74	98.52	9.61	8.87
Zolpidem	1	73.61	62.11	4.73	9.23
	10	71.5	108.6	6.38	6.44
	100	104.10	108.1	6.91	7.64
Zca	1	80.3	96.37	3.97	12.12
	10	86.63	85.2	5.31	7.36
	100	94.19	90.89	4.35	6.88
Zpca	1	106.52	111.67	6.72	8.59
	10	101.59	92.37	2.65	10.02
	100	95.15	82.60	6.99	9.21
Zopiclone	2	93.86	80.64	8.48	14.42
	10	86.90	84.01	4.99	7.49
	100	104.3	107.1	8.23	8.69
Zopiclone N-oxide	2	86.31	84.37	1.31	10.68
	10	84.79	85.41	5.51	9.05
	100	103.2	102.1	8.23	9.05
N-desmethylzopiclone	1	90.29	95.79	1.61	14.72
	10	109.52	86.41	4.88	8.58
	100	103.7	108.4	2.01	8.76
Melatonin	1	87.29	67.41	7.8	15.04
	10	83.33	82.36	6.4	11.39
	100	86.98	89.35	5.5	12.45
6-hydroxymelatonin	1	67.36	77.54	8.6	8.89
	10	77.71	82.36	6.9	7.33
	100	71.35	75.29	6.3	7.42
Alprazolam	2	92.67	102.89	6.43	8.62
	10	72.48	90.65	3.93	3.88
	100	94.37	97.17	5.43	12.46
A-hydroxyalprazolam	5	85.48	95.52	6.77	9.86
	10	100.3	111.97	8.89	10.10
	100	113.4	115.14	1.62	8.88
Triazolam	1	99.6	80.8	4.02	12.38
	10	99.06	121.1	5.81	10.34
	100	86.38	100.8	4.63	6.42
A-hydroxytriazolam	5	89.73	109.42	8.56	15.32
	10	75.17	99.0	9.25	14.02
	100	102.2	105.5	4.92	14.96

Midazolam	1	100.0	103.5	2.79	10.95
	10	88.32	94.0	1.37	12.44
	100	86.94	81.7	4.62	12.06
A-hydroxymidazdam	2	84.18	84.5	6.88	6.76
	10	94.42	90.4	5.24	12.06
	100	90.56	80.3	2.24	7.65
Clonazepam	5	90.35	94.2	7.28	8.38
	10	98.37	86.4	7.97	9.17
	100	95.02	99.0	4.29	6.05
7-aminoclonazepam	2	100.4	95.1	5.26	4.93
	10	99.5	98.9	7.15	4.02
	100	96.4	109.4	9.41	7.05
Diazepam	1	105.44	87.5	2.53	8.36
	10	101.4	99.7	1.71	6.02
	100	98.46	97.5	1.12	5.47
Nordazepam	2	99.17	95.1	4.27	7.53
	10	95.07	94.0	3.66	7.17
	100	93.85	89.4	4.25	6.57
Flunitrazepam	1	102.7	91.8	3.82	8.38
	10	92.0	88.5	6.45	9.17
	100	104.0	111.0	3.01	6.05
7-aminoflunitrazepam	1	84.65	92.1	11.8	4.93
	10	60.22	99.4	4.26	4.02
	100	85.58	101.2	6.71	7.05
Nitrazepam	1	104.8	82.6	2.40	8.05
	10	98.50	125.7	10.0	11.11
	100	91.70	119.8	0.62	5.20
7-aminonitrazepam	1	102.3	143.0	2.99	10.45
	10	106.8	128.1	1.54	4.80
	100	108.5	112.0	3.32	7.39
Temazepam	1	97.69	95.43	2.79	12.61
	10	92.24	88.1	1.37	9.18
	100	107.46	104.6	4.62	6.55
Estazolam	2	76.32	70.17	3.82	5.03
	10	90.37	86.95	5.20	6.69
	100	80.79	87.05	3.65	8.82
Chlordiazepoxide	2	72.79	61.88	8.99	7.99
	10	65.03	73.91	9.74	12.29
	100	79.33	88.10	1.4	9.59
Chlorpromazine	1	97.54	126.1	2.20	14.01
	10	97.08	86.2	1.30	9.70
	100	90.14	99.1	4.23	14.63

In this study, ESI (+) ionization mode was used and the mixed targets were injected respectively. A parent ion scan of m/z from 100 to 400 was conducted and the parent ion number $[M+H]^+$ was obtained from the generated first-order full scan spectra. The instrument parameters were adjusted using $[M+H]^+$ as the parent ion to perform second-order fragmentation analysis. As the collision energy increased, ion fragments gradually increased as well. Finally, the two daughter ions with the strongest signals were paired with respective parent ions, as

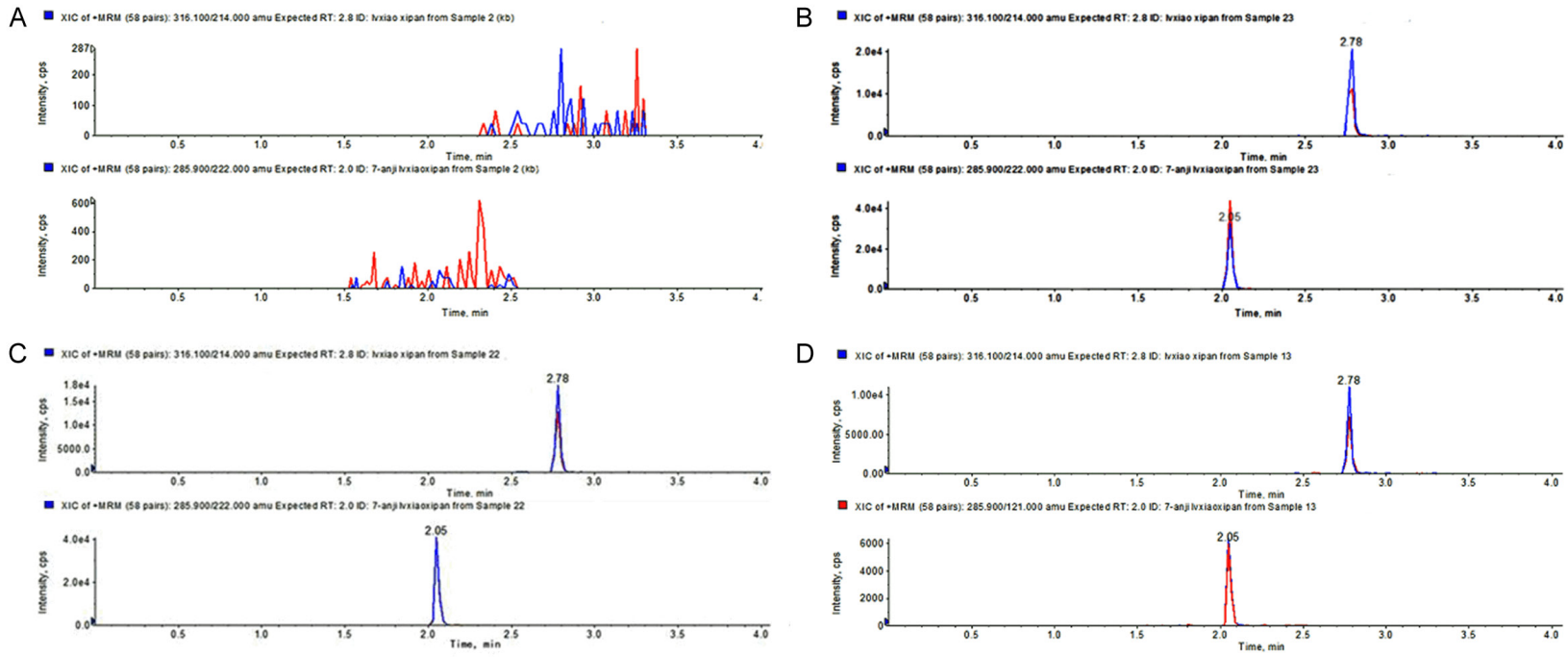


Figure 2. Toxicology screening MRM chromatogram of a victim's blood sample. A: Blank blood sample; B: Standard; C: Standard addition; D: The victim's blood sample.

the indicator of qualitative analysis, and the pair with the strongest signal was used as the indicator of quantitative analysis. Meanwhile, the declustering potential, the collision energy, and other parameters were also optimized. The targets were analyzed under selected conditions.

In April of 2016, a person went to dinner with a friend, and a few minutes later said person was in a coma. The next day, the victim woke up to discover she had been raped, and called the police. The police sent the victim's blood sample for testing within 24 hours. Clonazepam (26.77 ng/mL, tR=2.80 min, quantitative ion m/z 214.000) and the metabolite 7-aminoclonazepam (7.50 ng/mL, tR=2.09 min, quantitative ion m/z 121.000) were detected in the victim's blood. The reliability of these results was validated by spiking experiments, as shown in **Figure 2**. The results demonstrated that this method can be used for the analysis of real samples.

In conclusion, by optimizing chromatography, mass spectrometry conditions, and pretreatment methods, this study established a QuEChERS-UPLC-TQ/MS analysis method for these 29 drugs and their metabolites in whole blood. Our optimized QuEChERS pretreatment method takes advantage of simple and rapid protein removal via filtration and the low matrix effects of solid phase extraction, ensuring a high recovery rate while effectively reducing matrix effects. Our proposed method is fast-acting and sensitive, exhibits a high recovery rate, and can meet the needs of actual, real-world handling.

Acknowledgements

This work was supported by The Ministry of Public Security (201302ZDYJ002) and People's Public Security University of China (2019JKF223).

Disclosure of conflict of interest

None.

Address correspondence to: Yanjun Zhao, Department of Pharmacy, The First Affiliated Hospital of Zhejiang Chinese Medical University, No. 54 Youdian Road, Shangcheng District, Hangzhou 310006, Zhejiang Province, China. Tel: +86-13758162092; E-mail: zhaoyanjun99a@163.com; Leiping Zhang, Institute of Forensic Science, Ministry of Public Security, Muxidi Land, Xicheng District, Beijing 100032, China. Tel: +86-15801266364; E-mail: zhangleiping99a@163.com

References

[1] Kapil V, Green JL, Le Lait C, Wood DM and Dargan PI. Misuse of benzodiazepines and Z-drugs in the UK. Br J Psychiatry 2014; 205: 407-8.

[2] Pollmann AS, Murphy AL, Bergman JC and Gardner DM. Deprescribing benzodiazepines and Z-drugs in community-dwelling adults: a scoping review. BMC Pharmacol Toxicol 2015; 16: 19.

[3] Vårdal L, Wong G, Øiestad ÅML, Pedersen-Bjergaard S, Gjelstad A and Øiestad EL. Rapid determination of designer benzodiazepines, benzodiazepines, and Z-hypnotics in whole blood using parallel artificial liquid membrane extraction and UHPLC-MS/MS. Anal Bioanal Chem 2018; 410: 4967-4978.

[4] Montenarh D, Hopf M, Maurer HH, Schmidt P and Ewald AH. Detection and quantification of benzodiazepines and Z-drugs in human whole blood, plasma, and serum samples as part of a comprehensive multi-analyte LC-MS/MS approach. Anal Bioanal Chem 2014; 406: 803-18.

[5] Kapil V, Green JL, Le Lait C, Wood DM and Dargan PI. Misuse of benzodiazepines and Z-drugs in the UK. Br J Psychiatry 2014; 205: 407-8.

[6] Li PP, Zhang Y, Wu XY, Yang XF and Zhang ZS. Determination of 19 pesticides residues in seedless-litchi by gas chromatography-tandem mass spectro. Chinese Journal of Tropical Crops 2017; 38: 1565-1571.

[7] Rodríguez-González N, Uzal-Varela R, González-Castro MJ, Muniategui-Lorenzo S and Beceiro-González E. Determination of 9 triazine herbicides in aquatic products by GPC-SPE-GC-MS/MS. Environ Sci Pollut Res Int 2017; 24: 7764-7775.

[8] Ma X, Gao N and Chen B. Detection of geosmin and 2-methylisoborneol by liquid-liquid extraction-gas chromatograph mass spectrum (LLE-GCMS) and solid phase extraction-gas chromatograph mass spectrum (SPE-GCMS). Front Environ Sci Eng 2007; 1: 286-291.

[9] Xu GF, Nie JY, Li HF, Yan Z and Li J. Determination of 31 organophosphorous pesticide residues in fruits by gas chromatography with QuEChE. Journal of Instrumental Analysis 2016; 35: 1021-1025.

[10] Plassmann MM, Schmidt M, Brack W and Krauss M. Detecting a wide range of environmental contaminants in human blood samples-combining QuEChERS with LC-MS and GC-

MS methods. Anal Bioanal Chem 2015; 407: 7047-54.
[11] Cao X, Li X, Li J, Niu Y, Shi L, Fang Z, Zhang T and Ding H. Quantitative determination of carcinogenic mycotoxins in human and animal biological matrices and animal-derived foods using multi-mycotoxin and analyte-specific high performance liquid chromatography-tandem mass spectrometric methods. J Chromatogr B Analyt Technol Biomed Life Sci 2017; 1073: 191-200.
[12] Cuypers E, Vanhove W, Gotink J, Bonneure A, Van Damme P and Tytgat J. The use of pesticides in Belgian illicit indoor cannabis plantations. Forensic Sci Int 2017; 277: 59-65.
[13] Ruizgarcía Y, Silva CL and Gómezplaza E. A powerful analytical strategy based on QuEChERS-dispersive solid-phase extraction combined with ultrahigh pressure liquid chromatography for evaluating the effect of elicitors on biosynthesis of trans-resveratrol in grapes. Food Analytical Methods 2016; 9: 670-679.
[14] Custodio-Mendoza JA, Lorenzo RA, Valente IM, Almeida PJ, Lage MA, Rodrigues JA and Carro AM. Development of a partitioned liquid-liquid extraction-dispersive solid phase extraction procedure followed by liquid chromatography-tandem mass spectrometry for analysis of 3-monochloropropane-1,2-diol diesters in edible oils. J Chromatogr A 2018; 1548: 19-26.
[15] Eom T, Cho HD, Kim J, Park M, An J, Kim M, Kim SH and Han SB. Multiclass mycotoxin analysis in edible oils using a simple solvent extraction method and liquid chromatography with tandem mass spectrometry. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2017; 34: 2011-2022.
[16] Xian Y, Dong H, Wu Y, Guo X, Hou X and Wang B. QuEChERS-based purification method coupled to ultrahigh performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) to determine six quaternary ammonium compounds (QACs) in dairy products. Food Chem 2016; 212: 96-103.
[17] Alves V, Conceição C, Gonçalves J, Teixeira HM and Câmara JS. Improved analytical approach based on QuECHERS/UHPLC-PDA for quantification of fluoxetine, clomipramine and their active metabolites in human urine samples. J Anal Toxicol 2017; 41: 45-53.
[18] Xiang JJ, Liu XH and Xu G. Determination methods of benzodiazepines. Ningbo Chemical Industry 2014; 6-9.
[19] Matuszewski BK, Constanzer ML and Chavez-Eng CM. Matrix effect in quantitative lc/ms/ms analyses of biological fluids: a method for determination of finasteride in human plasma at picogram per milliliter concentrations. Anal Chem 1998; 70: 882-9.
[20] Kaczyński P. Clean-up and matrix effect in LC-MS/MS analysis of food of plant origin for high polar herbicides. Food Chem 2017; 230: 524.
[21] Yao M, Ma B and Ma Y. Overview of the matrix effect in quantitative liquid chromatography/mass spectrometry analyses of biological samples. J Pharm Anal 2010.
[22] Dmitrovic J, Chan SC and Chan SH. Analysis of pesticides and PCB congeners in serum by GC/MS with SPE sample cleanup. Toxicol Lett 2002; 134: 253-8.
[23] Xue YJ, Liu J and Pursley J. A 96-well single-pot protein precipitation, liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the determination of muraglitazar, a novel diabetes drug, in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci 2006; 831: 213-222.
[24] Howe PE. The use of sodium sulfate as the globulin precipitant in the determination of proteins in blood. J Biol Chem 1921; 49: 93-107.