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Research Article

Direct quantitative analysis of benzodiazepines, metabolites, and analogs in diluted human urine by rapid resolution liquid chromatography tandem mass spectrometry

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ABSTRACT

Rapid resolution liquid chromatography (RRLC) coupled with triple quadrupole mass spectrometry (QqQ-MS) was developed for direct quantitative analysis of benzodiazepines (BDZs) and their metabolites, as well as BDZ analogs (zopiclone, zolpidem, and zaleplon) in diluted human urine. A perfluorophenyl column packed with sub-2-μm particles and a C8 precolumn were applied to accomplish RRLC analysis with symmetric peak shapes. Urine samples were diluted 10-fold with Milli-Q water prior to autoinjection for direct quantification of 34 target analytes with negligible matrix effect. Gradient elution and dynamic multiple reaction monitoring were used for simultaneous quantitation of 34 BDZs, metabolites, and BDZ analogs. Good recovery was obtained in the range of 80.2–98.5% and the limit of detection ranged from 0.01 ng/mL to 0.5 ng/mL for all 34 target analytes in spiked urine. Moreover, good precision and accuracy were obtained for quantitative determination in diluted urine samples by the proposed RRLC/QqQ-MS method for intra-day/inter-day assays in the ranges of 0.1–8.8%/0.1–8.9% and 91.2–106.1%/89.6–104.6%, respectively. The applicability of this newly developed RRLC/QqQ-MS method was demonstrated by quantitative determination of BDZs, metabolites, and BDZ analogs in various clinical urine samples.

1. Introduction

Benzodiazepines (BDZs) and their analogs, such as zopiclone, zolpidem and zaleplon, have been used as prescribed medications for treating anxiety, insomnia, or muscle spasm after they became commercially available since the 1960s [1,2]. Although BDZs were considered to have low toxicity, the potential of addiction or dependence has still received much

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attention from time to time [2,3]. In addition, abuse of BDZs and some related substances was found to be associated with suicide or drug-facilitated sexual assault. In many cases, the drug-facilitated sexual assault victims might not report the event and receive medical attention until several hours or days after the incident. Therefore, a rapid and sensitive analytical method became desirable to determine quantitatively the trace residual BDZs and/or metabolites in biological fluids, such as urine, and provide valuable information for clinical diagnosis as well as forensic applications [4–8].

Several publications have reported the quantitation of BDZs and related substances in biological samples including hair, blood, and urine by various analytical techniques. Several research groups reported the use of immunoassays for determining BDZs in either hair or urine samples, however, cross-reactions were frequently observed and other chromatographic methods were usually required to determine quantitatively individual BDZs [9,10]. Miller et al [9] reported that crossreactivity of various BDZs ranged from 8% to 143% for microplate enzyme-linked immunosorbent assay directed towards oxazepam, due to similar structures or common crossreacting epitopes of BDZs and metabolites. The commonly used chromatographic methods for quantifying BDZs in biological samples included gas chromatography (GC) coupled to mass spectrometry (MS) [6,10,11] and liquid chromatography (LC) with UV [12–14] or MS detection [4,7,15–22]. In most GC–MS methods, derivatization and sample pretreatment process, such as liquid–liquid extraction (LLE), were often required for simultaneous quantification of BDZs in biological samples [6,10]. Recently, ultra-performance liquid chromatography (UPLC) or rapid resolution liquid chromatography (RRLC) coupled to MS was used for simultaneous quantitation of multiple BDZ-related substances in biological samples [5,15,20]. Compared with conventional high-performance liquid chromatography (HPLC)–MS methods, UPLC–MS or RRLC–MS offers the advantages of rapid analysis and enhanced sensitivity for quantitative determination [5,15,20,21]. Although LC–MS methods were considered to be suitable for quantifying BDZs in biological samples without derivatization, sample pretreatment procedures such as LLE [21,22], solid phase extraction (SPE) [4,7,15–17], or molecularly imprinted SPE [18,19] were generally involved in order to reduce the matrix effect and to achieve sensitive detection. For instance, Verplaetse et al [15] claimed that the limit of detection (LOD) of 29 BDZs and BDZ-like hypnotics in urine ranged from 0.02 ng/mL to 0.2 ng/mL using SPE prior to UPLC–tandem MS analysis. Ariffin and colleagues [18] used molecularly imprinted SPE for extracting BDZs and metabolites from hair samples and reported the LODs and limits of quantitation (LOQs) in the range of 0.03–0.78 ng/mL and 0.06–1.32 ng/mL, respectively.

In this study, a RRLC/triple quadrupole MS (QqQ-MS) system was established for direct quantitation of BDZs, metabolites, and BDZ analogs (zopiclone, zolpidem, and zaleplon) in dilute human urine without tedious sample pretreatment procedures. The experimental conditions for MS detection and RRLC analysis were optimized prior to evaluating the proposed RRLC/QqQ-MS method for quantitative screening of BDZ metabolites and BDZ analogs. The following experiments, including dynamic multiple reaction monitoring (dMRM) parameters for QqQ-MS detection, the effect of formic acid in mobile phase, the use of precolumn prior to the RRLC separation, as well as minimizing the matrix effect by dilution and postcolumn addition, were performed to achieve the optimal conditions for analyzing 34 BDZs and related substances. The quantitative performance of the proposed RRLC/QqQ-MS method was validated by obtaining the linear range, precision, and accuracy, as well as the LOD and LOQ of individual analytes under the optimal conditions. Clinical urine samples were diluted with Milli-Q water and then directly quantified by RRLC/QqQ-MS to demonstrate the applicability of the proposed RRLC/QqQ-MS method for clinical applications.

2. Methods

2.1. Materials and reagents

Internal standards (ISTD-1: zolpidem-d_6 and ISTD-2: prazepam-d_5), 7-aminonitrazepam, 7-aminoclonazepam, 4-hydroxynordiazepam, 7-aminoflunitrazepam, zopiclone, bromazepam, chlordiazepoxide, zolpidem, α-hydroxymidazolam, oxazepam, α-hydroxyalprazolam, α-hydroxytriazolam, trazodone, nitrazepam, lorazepam, zaleplon, *N*-desmethylflunitrazepam, 2-hydroxyethylflurazepam, nordiazepam, estazolam, midazolam, clonazepam, desalkylflurazepam, temazepam, flurazepam, lormetazepam, nimetazepam, flunitrazepam, clobazam, alprazolam, triazolam, brotizolam, diazepam, and prazepam were purchased from Cerilliant Corp. (Austin, TX, USA). HPLC-grade methanol and acetonitrile were obtained from Mallinckrodt Baker, Inc. (Paris, KY, USA). Formic acid (98–100%) and ammonium formate were purchased from Riedel-de Haen (Seelze, Germany). Deionized water was produced from a Milli-Q Integral 5 water purification system (Millipore, Bedford, MA, USA). Drug-free urine samples were collected from voluntary healthy adults for the study.

2.2. Instrumentation and LC conditions

Chromatographic separation was carried on an Agilent 1200 Series RRLC system (Agilent, Waldbronn, Germany), including an online degasser, a binary pump, an autosampler, equipped with a Zorbax SB-C8 pre-column (2.1 mm × 30 mm, 3.5 μm; Agilent) and a Hypersil GOLD perfluorophenyl (PFP) column (2.1 mm × 100 mm, 1.9 μm; Thermo Scientific, Waltham, MA, USA) in an Agilent Thermostatted Column Compartment (set at 40 °C). Gradient elution was used during the separation process. The mobile phase consisted of two solvents: solvent A was Milli-Q water containing 0.1% formic acid and 1mM ammonium formate; and solvent B was acetronitrile containing 0.1% formic acid and 1mM ammonium formate. Starting from 10% B, the mobile phase was changed to 20% B in 3 minutes, then to 40% B in a further 5 minutes, and to 70% B in an additional 4 minutes. Afterwards, the mobile phase was immediately raised to 99% B for 1.5 minutes and the LC column was re-equilibrated for 3.5 minutes using 10% B. The flow rate was set at 0.3 mL/min.

An Agilent 6410 Triple quad LC/MS mass spectrometer was used for QqQ-MS detection under the positive mode with electrospray ionization source at a spray voltage of 4000 V.

Heated N_2 gas (10 L/min at 350 °C) served as drying gas to evaporate solvent from the ionization chamber. System control, data acquisition, and data processing for RRLC/QqQ-MS analysis were performed using Agilent Mass Hunter Workstation (version B.02.01) software. dMRM (time window: retention time ± 1 min) [23,24] was utilized for QqQ-MS measurement.

2.3. *Urine sample preparation*

One milliliter of urine sample containing two ISTDs (2 ng/mL each) was diluted 10-fold with Milli-Q water. About 1 mL of clean supernatant was transferred to the autosampler sample vial. Then, 20 μL of the pretreated sample solution was auto-injected for RRLC/QqQ-MS analysis.

2.4. *Validation process*

The following experiments were performed to validate the proposed method for quantitative analysis of BDZs, metabolites, and analogs in human urine. A calibration curve, peak area ratios (analyte/ISTD) versus concentration, was established for each analyte to evaluate the linearity by the proposed RRLC/QqQ-MS method for quantitative screening. Calibration standards (0.1 ng/mL, 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, and 500 ng/mL) containing two ISTDs (2 ng/mL each) were prepared by spiking different amounts of analytes into drug-free urine to make the standard mixtures with desired concentrations. The spiked urine standards were then diluted 10 times with Milli-Q water prior to RRLC/QqQ-MS analysis. Measurements of each standard solution were carried out in triplicate and the LOD and LOQ were determined using the single-to-noise ratio of 3 and the lowest concentration in the linear range, respectively. The precision and accuracy, including intra-day and inter-day experiments, were evaluated using three different concentrations of spiked urine samples. Quantitation of all target analytes was determined by the peak area ratios (A_{anal}/A_{ISTD}), where A_{anal} is the peak areas of individual analytes and A_{ISTD} is the peak areas of the corresponding ISTD (zolpidem-d_6 or prazepam-d_5). As shown in Table 1, analytes 1–16 and 17–34 were determined using zolpidem-d_6 and prazepam-d_5 as the corresponding ISTD, respectively.

Table 1 – MRM parameters for RRLC/QqQ-MS analysis under positive mode.

		$[M+H]^+$	Monitored ions	Fragmentor (V)	Collision energy (V)
1	7-Aminonitrazepam	252	121[a]/94/74	144	24/40/64
2	7-Aminoclonazepam	286	250/222/121[a]	144	16/24/28
3	4-Hydroxynordiazepam	287	165[a]/140/77	144	28/28/72
4	7-aminoflunitrazepam	284	135[a]/93/77	144	28/60/80
5	Zopiclone	389	245[a]/217/112	92	12/36/72
6	Bromazepam	316	208/182[a]/154	144	40/36/76
7	Chlordiazepoxide	300	282[a]/227/165	92	20/20/56
8	Zolpidem	308	263/235[a]/65	144	24/36/80
	Zolpidem-d_6 (ISTD-1)	314	263/235[a]/65	144	24/36/80
9	α-Hydroxymidazolam	342	324[a]/203/168	144	20/24/44
10	Oxazepam	287	269/241[a]/77	92	8/20/68
11	α-Hydroxyalprazolam	325	297[a]/216/205	144	24/44/48
12	α-Hydroxytriazolam	359	331[a]/239/176	144	28/28/48
13	Trazodone	372	176[a]/148/78	144	24/36/64
14	Nitrazepam	282	236[a]/207/180	144	24/36/40
15	Lorazepam	321	303/275[a]/229	92	12/21/36
16	Zaleplon	306	264/236[a]/64	144	20/24/80
17	N-Desmethylflunitrazepam	300	254[a]/225/198	144	24/36/40
18	2-Hydroxyethylflurazepam	333	211/140/109[a]	144	36/44/28
19	Nordiazepam	271	165/140[a]/77	144	28/28/60
20	Estazolam	295	267[a]/205/151	144	20/44/72
21	Midazolam	326	291[a]/249/209	144	28/40/36
22	Clonazepam	316	270[a]/214/151	144	24/40/76
23	Desalkyflurazepam	289	226/140[a]/77	144	28/32/72
24	Temazepam	301	255[a]/193/177	92	20/36/40
25	Flurazepam	388	317/315[a]/134	144	16/20/60
26	Lormetazepam	335	289[a]/177/75	92	20/44/80
27	Nimetazepam	296	250[a]/221/165	144	24/36/56
28	Flunitrazepam	314	268[a]/239/183	144	24/36/64
29	Clobazam	301	259[a]/224/77	92	16/32/60
30	Alprazolam	309	281[a]/205/151	144	24/44/76
31	Triazolam	343	315/308[a]/239	144	28/24/44
32	Brotizolam	393	314[a]/279/210	144	20/28/44
33	Diazepam	285	193[a]/154/89	144	32/28/80
34	Prazepam	325	271[a]/165/140	144	20/40/40
	Prazepam-d_5 (ISTD-2)	330	276[a]/165/140	144	20/40/40

MRM = multiple reaction monitoring; QqQ-MS = triple quadrupole mass spectrometry; RRLC = rapid resolution liquid chromatography.
[a] Quantitative ion.

3. Results and discussion

3.1. Optimization of RRLC/QqQ-MS conditions

Using the condition mentioned in *Urine sample preparation* and Table 1, the same precursor ions ($[M+H]^+$) or transitions of target analytes/ISTDs reported in various publications [4,15–17,20,22,25] were detected. Therefore, the $[M+H]^+$ ion was chosen as the precursor ion for QqQ-MS measurements. The characteristic fragment ions for identification and quantitative determination of each analytical component were summarized in Table 1 to conduct further experiments.

Due to the complex chemical structures of BDZs [5], a PFP column was utilized for analyzing BDZs and related substances in the present study. Previous publications showed that this type of packing material was suitable for halogenated compounds as well as analytes with a phenyl ring and/or a fused ring system [26,27]; however, using only a PFP column resulted in asymmetric peak shapes for some analytes as shown in Fig. 1A. The peak shapes were greatly improved, as shown in Fig. 1B, whereas an additional C8 column was applied prior to the PFP column.

Furthermore, improved LC resolution and decreased MS sensitivity were observed by the increasing amounts of formic acid (0.1%, 0.2%, and 0.5%) in the mobile phase. Therefore, 0.1% formic acid was added to the mobile phase to achieve better LC resolution with a small (~10%) decrease in sensitivity. The representative chromatograms of spiked urine (10 ng/mL each) are shown in Fig. 2. Under optimized UPLC elution conditions, the analysis of 34 BDZs could be completed within 13 minutes. As a result, this UPLC method can analyze more BDZs in less time compared to the reported LC methods [9,18].

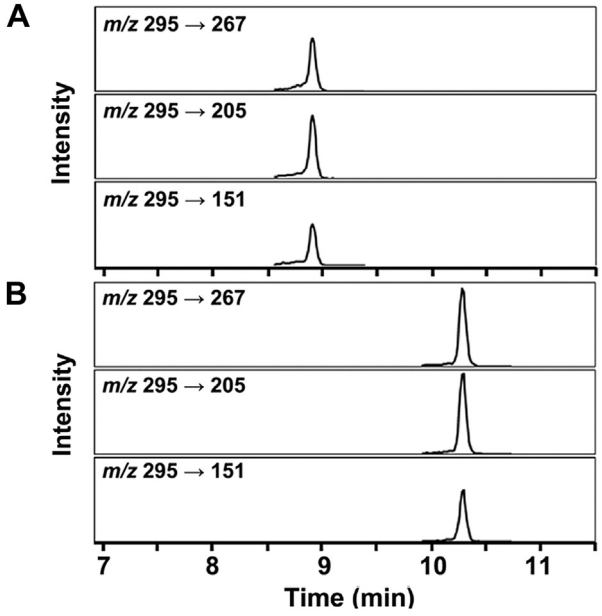

Fig. 1 – Peak shape of target analytes (10 ng/mL, eastazolam as the example) in spiked Milli-Q water without (A) or with (B) the use of C8 precolumn prior to the perfluorophenyl column.

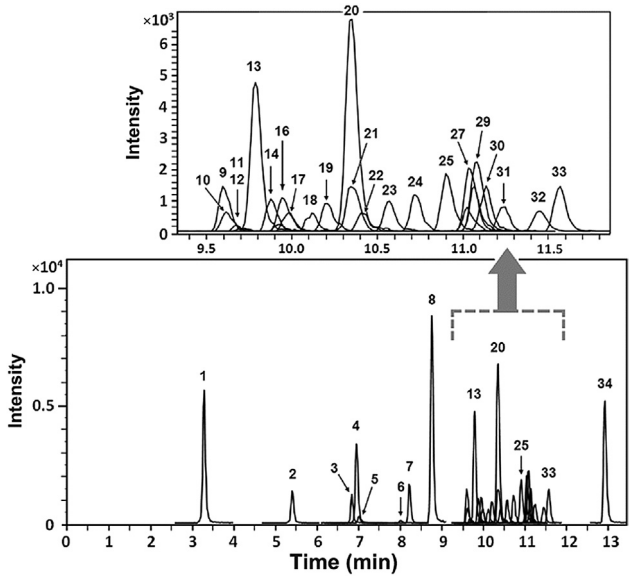

Fig. 2 – Representative chromatograms of benzodiazepine-spiked (10 ng/mL) urine sample. Sample was diluted 10-fold by Milli-Q water prior to rapid resolution liquid chromatography/triple quadrupole mass spectrometry analysis. The peak identification is shown in Table 1.

3.2. Matrix effect in human urine

Diluted urine samples were used for direct analysis by RRLC/QqQ-MS to avoid the laborious sample pretreatment procedures such as LLE and SPE. BDZ-spiked (10 ng/mL each) urine samples diluted five-fold, 10-fold, and 20-fold using Milli-Q water were utilized for evaluating the effect of dilution factor on the recovery/yield. The average recoveries ($n = 3$) of the total 34 analytes in five-fold, 10-fold, and 20-fold diluted urines were 74.7%, 86.7%, and 90.0%, respectively. In five-fold diluted urine samples, there were 25 analytes with recoveries <80% and 11 analytes with recoveries <70%. All analytes in both 10-fold and 20-fold diluted urine samples had recoveries in the ranges of 80.2–98.5% and 80.9–98.9%, respectively. Although higher recoveries were found in 20-fold dilute urine samples, we used 10-fold diluted urine for the subsequent experiments to achieve better sensitivity in quantitative measurement.

To study further the matrix effect during a complete chromatographic run, the postcolumn infusion [28,29] of a standard mixture in Milli-Q water was used to monitor the matrix interference in RRLC/QqQ-MS analysis of urine samples. Using an auxiliary syringe pump, a standard mixture (50 ng/mL each) was delivered and joined (through a T-junction) into the analytical effluent before the electrospray for MS detection. As shown in Fig. 3, a noticeable matrix effect caused by the endogenous components in diluted urine sample was observed in high aqueous conditions (analysis time 1–2 minutes), whereas little influence on MS signals was found after 2 minutes throughout the rest of the LC analysis. The use of diluted urine was confirmed suitable for direct analysis of BDZs, metabolites, and BDZ analogs by RRLC/QqQ-MS, with negligible matrix effect.

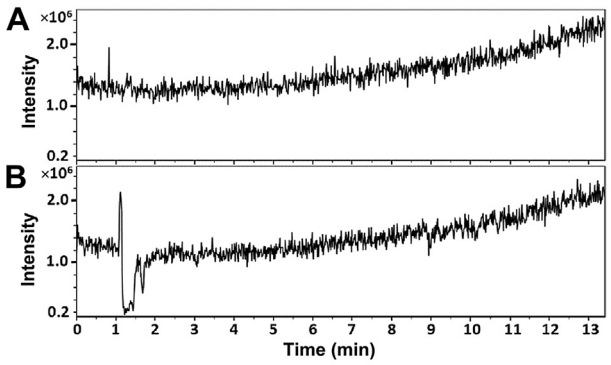

Fig. 3 – Matrix effect of diluted urine (10-fold dilution) on mass spectrometry signals using postcolumn infusion. Infusion profiles for a standard mixture (50 ng/mL each) after injection of (A) Milli-Q water and (B) dilute urine.

3.3. *Quantitative performance*

The validation of applying RRLC/QqQ-MS for direct quantitative determination of BDZs, metabolites, and BDZ analogs was achieved by examining the linearity, LOD, LOQ, as well as precision and accuracy using spiked urine samples under optimal conditions. The merits of quantitative measurement (linearity, LOD and LOQ) of individual analytes are summarized in Table 2. In the total 34 target analytes, more than two-thirds of them had a linear range around three orders of magnitude and only two analytes (trazodone and flurazepam) less than two orders. The LODs of all analytes were determined to be ≤0.5 ng/mL and half of them had LODs in the range of 0.01–0.05 ng/mL. The precision and accuracy of the proposed method were also investigated and the results are summarized in Table 3. Both intra-day and inter-day experiments were performed at three concentration levels. The ranges of precision for intra-day and inter-day assays were 0.1–8.8% and 0.1–8.9%, respectively. The corresponding accuracy ranges were 91.2–106.1% and 89.6–104.6% for intra-day and inter-day experiments, respectively. Based on these

Table 2 – Retention time, linear range, LOD, and LOQ of benzodiazepines and metabolites by rapid resolution liquid chromatography/ triple quadrupole mass spectrometry analysis.

		Retention time (min)	Linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
1	7-Aminonitrazepam	3.28	0.1–500.0	0.025	0.1
2	7-Aminoclonazepam	5.38	0.1–500.0	0.05	0.1
3	4-Hydroxynordiazepam	6.83	0.1–250.0	0.05	0.1
4	7-Aminoflunitrazepam	6.94	0.1–500.0	0.05	0.1
5	Zopiclone	7.05	1.0–250.0	0.25	1.0
6	Bromazepam	8.01	1.0–500.0	0.50	1.0
7	Chlordiazepoxide	8.23	0.1–500.0	0.05	0.1
8	Zolpidem	8.79	5.0–500.0	0.01	5.0
9	α-Hydroxymidazolam	9.62	0.1–500.0	0.05	0.1
10	Oxazepam	9.63	0.5–500.0	0.25	0.5
11	α-Hydroxyalprazolam	9.67	1.0–500.0	0.25	1.0
12	α-Hydroxytriazolam	9.71	5.0–500.0	0.50	5.0
13	Trazodone	9.81	10.0–500.0	0.025	10.0
14	Nitrazepam	9.89	0.5–500.0	0.10	0.5
15	Lorazepam	9.94	1.0–500.0	0.50	1.0
16	Zaleplon	9.96	0.1–500.0	0.10	0.1
17	N-Desmethylflunitrazepam	10.00	0.5–500.0	0.25	0.5
18	2-Hydroxyethylflurazepam	10.13	0.5–500.0	0.25	0.5
19	Nordiazepam	10.20	0.5–500.0	0.25	0.5
20	Estazolam	10.37	0.1–500.0	0.01	0.1
21	Midazolam	10.37	5.0–500.0	0.10	5.0
22	Clonazepam	10.43	0.5–500.0	0.25	0.5
23	Desalkyflurazepam	10.57	0.5–500.0	0.10	0.5
24	Temazepam	10.73	0.5–500.0	0.10	0.5
25	Flurazepam	10.94	0.5–5.0	0.025	0.5
26	Lormetazepam	11.04	0.5–100.0	0.25	0.5
27	Nimetazepam	11.06	0.1–500.0	0.05	0.1
28	Flunitrazepam	11.09	0.1–500.0	0.05	0.1
29	Clobazam	11.09	0.1–500.0	0.05	0.1
30	Alprazolam	11.13	0.1–500.0	0.05	0.1
31	Triazolam	11.24	0.1–500.0	0.05	0.1
32	Brotizolam	11.45	0.5–500.0	0.10	0.5
33	Diazepam	11.56	0.1–500.0	0.05	0.1
34	Prazepam	12.93	0.5–500.0	0.025	0.5

LOD = limit of detection; LOQ = limit of quantitation.

Table 3 – Precision and accuracy at three concentration levels for the quantitative determination of 34 target analytes.

	Concentration level[a]	Intra-day ($n = 3$)		Inter-day ($n = 3$)	
	(ng/mL)	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)
7-Aminonitrazepam	0.5/10.0/250.0	1.0/0.3/0.4	100.1/99.9/100.1	2.1/1.6/2.7	100.9/101.3/97.8
7-Aminoclonazepam	0.5/10.0/250.0	2.1/1.9/2.2	99.5/100.7/100.1	2.4/0.5/1.8	98.4/101.0/100.1
4-Hydroxynordiazepam	0.5/10.0/100.0	4.0/3.1/6.8	105.0/100.4/100.1	7.9/0.7/1.9	97.6/101.2/101.6
7-aminoflunitrazepam	0.5/10.0/250.0	0.7/2.2/1.8	98.4/99.6/101.3	3.9/2.3/1.9	97.7/97.1/102.3
Zopiclone	5.0/10.0/100.0	1.5/6.4/8.5	100.5/105.4/99.0	1.6/2.5/0.4	99.7/102.5/99.1
Bromazepam	5.0/10.0/250.0	2.9/1.2/0.3	98.1/99.5/100.4	0.5/0.4/0.7	98.6/100.1/100.6
Chlordiazepoxide	0.5/10.0/250.0	1.5/1.9/1.8	94.3/100.9/100.3	2.3/2.1/0.7	95.6/100.1/101.1
Zolpidem	10.0/50.0/250.0	0.7/3.1/2.3	91.2.101.2/101.0	1.8/2.1/2.7	89.6/102.7/103.5
α-Hydroxymidazolam	0.5/10.0/250.0	3.7/0.3/2.4	101.3/99.7/99.1	2.4/0.1/0.1	102.3/99.5/99.1
Oxazepam	1.0/10.0/250.0	0.3/2.6/2.1	98.5/98.5/102.1	4.2/2.4/0.5	98.8/96.9/102.6
α-Hydroxyalprazolam	5.0/10.0/250.0	6.3/1.9/1.2	100.5/100.3/98.7	0.9/0.6/0.4	101.4/99.8/99.1
α-Hydroxytriazolam	10.0/50.0/250.0	3.8/7.7/0.9	100.5/98.9/92.4	5.1/1.5/4.7	98.2/100.6/97.5
Trazodone	50.0/100.0/250.0	0.9/1.7/0.6	104.4/100.7/98.3	3.7/4.8/4.6	100.4/98.1/101.9
Nitrazepam	1.0/10.0/250.0	4.7/0.8/6.7	98.9/100.0/100.2	1.8/0.9/0.2	97.9/100.1/100.1
Lorazepam	5.0//10.0/250.0	7.1/6.3/2.2	99.0/96.2/102.8	0.9/2.0/1.0	98.1/97.5/103.4
Zaleplon	0.5/10.0/250.0	2.2/1.3/2.7	100.5/98.8/100.2	6.8/2.4/4.2	96.3/96.4/104.6
N-Desmethylflunitrazepam	1.0/10.0/250.0	3.7/3.2/0.6	99.6/99.3/100.3	4.5/0.8/0.9	99.7/100.2/99.8
2-Hydroxyethylflurazepam	1.0/10.0/250.0	3.5/1.1/1.0	99.7/99.9/99.5	2.7/0.7/0.5	97.9/100.7/99.8
Nordiazepam	1.0/10.0/250.0	7.3/2.2/0.3	100.5/100.3/98.9	1.1/1.3/0.8	99.4/100.6/99.2
Estazolam	0.5/10.0/250.0	6.4/1.7/1.0	99.1/100.6/101.5	1.6/1.7/0.4	97.4/98.9/101.8
Midazolam	10.0/100.0/250.0	0.3/3.5/0.2	94.9/102.4/99.9	2.1/3.1/3.9	96.2/99.4/102.4
Clonazepam	1.0/10.0/250.0	8.6/0.7/1.6	106.1/98.6/98.3	8.9/1.9/1.7	98.2/100.7/99.7
Desalkylflurazepam	1.0/10.0/250.0	5.9/3.6/0.7	96.8/100.4/101.0	2.0/0.7/1.1	99.0/100.2/100.6
Temazepam	1.0/10.0/250.0	5.6/1.0/0.3	100.6/99.5/100.6	0.6/4.0/2.8	100.1/95.8/103.1
Flurazepam	0.5/1.0/5.0	0.7/2.5/5.2	99.7/100.7/99.6	0.8/3.9/2.3	99.0/103.7/98.0
Lormetazepam	1.0/10.0/50.0	8.8/1.3/3.4	98.8/102.1/102.2	1.7/1.4/2.6	100.1/101.0/99.6
Nimetazepam	0.5/10.0/250.0	3.7/1.9/2.5	101.2/99.3/101.4	5.2/1.0/0.4	96.7/100.4/101.0
Flunitrazepam	0.5/10.0/250.0	1.3/1.2/1.2	96.8/100.4/100.2	3.9/0.6/0.8	95.2/100.8/100.9
Clobazam	0.5/10.0/250.0	2.7/1.1/0.1	96.8/101.2/100.8	4.2/2.1/2.6	95.1/99.7/102.4
Alprazolam	0.5/10.0/250.0	7.0/0.3/1.1	101.2/99.3/99.2	3.8/0.7/3.6	97.7/98.8/102.1
Triazolam	0.5/10.0/250.0	2.1/0.4/0.7	97.8/100.7/100.5	2.7/1.6/1.2	97.2/99.3/101.2
Brotizolam	1.0/10.0/250.0	6.5/2.0/2.5	103.3/99.4/99.7	2.6/1.0/2.4	100.4/98.3/102.0
Diazepam	0.5/10.0/250.0	0.3/2.3/1.3	104.3/98.7/98.9	5.6/1.6/0.6	99.6/99.9/99.7
Prazepam	1.0/10.0/250.0	0.2/0.9/0.8	99.4/99.2/100.2	0.7/2.0/0.3	99.1/100.3/100.0

RSD = relative standard deviation.
[a] Three concentration levels (low/medium/high) were chosen depending on the linear range of individual analyte.

results, this RRLC/QqQ-MS method was verified to be suitable for direct quantitative screening of BDZs, metabolites, and BDZ analogs in diluted human urine. In addition, these results are comparable with other reported LC-MS/MS methods for analyzing BDZs and related substances in human urine. For instance, Marin et al [4] reported an LOD of 10 ng/mL for 13 BDZs, whereas Verplaetse's group [15] claimed that the LODs ranged from 0.02 ng/mL to 0.2 ng/mL for 29 BDZs and BDZ-like hypnotics in urine. Ishida et al [5] reported an LOD of 0.2–8.0 ng/mL for 43 BDZs and their metabolites by UPLC-MS/MS. However, all these studies involved a time-consuming pretreatment process that could take up to several hours prior to LC-MS/MS analysis.

3.4. *Application: Quantitative screening of multiple BDZ-related substances in human urine*

Several clinical urine samples from a local hospital were analyzed by the proposed RRLC/QqQ-MS method. Fig. 4 shows the representative chromatograms of a drug-free urine sample and a clinical urine sample. In this clinical sample, three compounds were identified and quantified based on the three MRM transitions of individual analytes shown in Table 1.

Compared with the urine blank and the BDZ-spiked samples, the unidentified peak shown in Fig. 4A could be the matrix components from the individual. Table 4 summarizes the identified compounds with their corresponding concentrations in the urine samples. For example, 7-aminoclonazepam, the major metabolite of clonazepam [2], was found in three clinical urine samples with concentrations ranging from 1.3 ng/mL to 79.3 ng/mL. Combined with personal background information as well as medication history, the ingested amounts, or the time periods of drug intake might be resolved and offer useful information for clinical diagnosis or giving proper medical treatments to patients.

In conclusion, we developed a rapid and sensitive RRLC/QqQ-MS method using a sub-2-μm packed PFP column for quantitative analysis of BDZs, metabolites, and BDZ analogs in diluted human urine. In this newly developed assay, only a minimal sample pretreatment procedure (dilution of urine sample) was needed; however, no significant deterioration on LC column performance was observed after >200 injections of

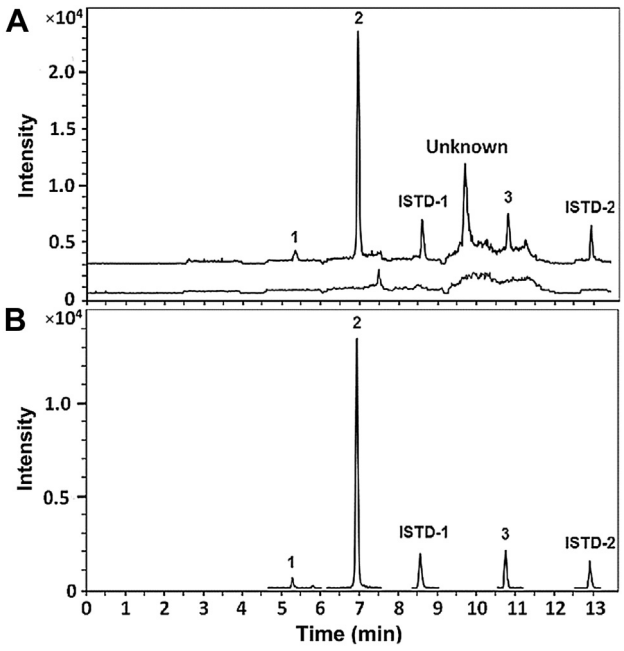

Fig. 4 – Representative chromatograms for a clinical urine sample, U4, presented in (A) TIC (urine blank at the bottom trace) and (B) dMRM results. Based on the dMRM results, three peaks were determined as follows. Peak 1: 7-aminoclonazepam (1.6 ng/mL); Peak 2: 7-aminoflunitrazepam (13.3 ng/mL); and Peak 3: flurazepam (4.9 ng/mL). TIC = total ion count; dMRM = dynamic multiple reaction monitoring.

urine samples. This method offered good linearity ($R^2 \geq 0.997$) as well as intra-day/inter-day precision and accuracy for quantitative determination of 34 target analytes in human urine. Compared with other LC-MS/MS methods to analyze BDZs and related substances in human urine, this RRLC/QqQ-MS method offered comparable sensitivity with LOD values of 0.01–0.05 ng/mL for 17 analytes and 0.1–0.5 ng/mL for the others, to determine quantitatively the 34 BDZs and related substances. We have demonstrated a newly developed rapid RRLC/QqQ-MS method with low LOD values as an alternative for high-throughput quantitative measurement of BDZs, metabolites, and BDZ analogs in human urine for clinical applications.

Table 4 – Quantitative screening of benzodiazepines and metabolites in clinical urine samples by the proposed rapid resolution liquid chromatography/triple quadrupole mass spectrometry method.

Sample no.	Identified compounds	Measured concentration (ng/mL)
U1	7-Aminoclonazepam	79.3
U2	Chlordiazepoxide	7.1
	Nordiazepam	2.9
U3	Oxazepam	2.2
U4	7-Aminoclonazepam	1.6
	7-aminoflunitrazepam	13.3
	Flurazepam	4.9
U5	7-Aminoclonazepam	1.3
	7-aminoflunitrazepam	32.2
U6	Flurazepam	1.8
U7	7-Aminonitrazepam	0.13
	Bromazepam	25.2
	Nordiazepam	0.6

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