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GC–MS quantification of ketamine, norketamine, and dehydronorketamine in urine specimens and comparative study using ELISA as the preliminary test methodology

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Abstract

An automated solid-phase extraction procedure combined with the gas chromatography–mass spectrometry (GC–MS) methodology, without derivatization, has been developed for the determination of ketamine (K), norketamine (NK), and dehydronorketamine (DHNK) in urine. The analytical approach is simple and rapid, yet reliable, achieving good linearity ($r^2 > 0.999$ over the concentration range of 30 to 1000 ng/mL), sensitivity (limits of quantification = 15, 10, and 20 ng/mL for K, NK, and DHNK, respectively), accuracy (90–104%), and precision (RSD < 8.1%) for all analytes. Two hundred and six urine specimens collected from suspected drug users were analyzed by this protocol and also screened by Neogen ELISA method to evaluate the efficiency as well as the compatibility of these two methods. Neogen ELISA showed high efficiency (98.1%), high sensitivity (90.9%), high specificity (98.9%), low false-positive rate (1.1%), and moderate false-negative rate (9.1%), adopting 10 ng/mL K as the cutoff. Neogen ELISA screening followed by GC–MS analysis appeared to be a good screening-confirmation test scheme for the analysis of K in urine. Twenty of the 22 positive urine specimens contained all three analytes simultaneously, with DHNK showing the highest and K the lowest concentrations.

Keywords: Ketamine; Norketamine; Dehydronorketamine; GC–MS; ELISA

1. Introduction

Club drugs are commonly used at rave parties, nightclubs, and music festivals to enhance sensory stimulation and social intimacy. They are inexpensive and easily distributed as small pills or in powder or liquid forms that are taken orally and often in combination with alcohol, or other drugs, to enhance their effect. Club drugs are especially popular among youngsters for recreation purpose.

Ketamine (K), one of the most widely used club drugs and a parenterally administered anesthetic agent, exhibits sedative, amnestic, and analgesic properties [1]. Abused at a higher dose than normally used for anesthetic purpose, ketamine generates effects similar to those produced by phencyclidine (PCP) with visual effects from lysergide (LSD) use. The over all self-administration behavior is similar to that exhibited by central nervous system depressant drugs in animal studies [2].

In recent years, the abuse of K has dramatically increased worldwide [3–5] requiring the development of effective screening and confirmation methods. Since 1970s, several analytical methods for the determination of K and its metabolites in human urine, plasma, and hair have been developed. These methods were based on gas chromatography with flame-ionization detector (GC-FID) [6], nitrogen–phosphorus detector (GC-NPD) [7], and high-performance liquid chromatography (HPLC) [8]. More specific methods using mass spectrometer as the detector, i.e. gas chromatography–mass spectrometry (GC–MS) [6,7,9–18] and liquid chromatography–mass spectrometry (LC–MS) methods [18,19], have been reported. More recently, highly specific approaches based on liquid chromatography–tandem mass spectrometry (LC–MS–MS) [20] and headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC–MS) [21] have also been advocated as effective "screening" methods.

While LC–MS approaches and GC–MS-based screening methodology may serve specific needs, two-step testing strategy, with each based on a different underlying principle, is

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currently considered the most effective approach under high-volume testing environment. With this in mind, this study has established a correlation of test data between the preliminary and the confirmatory test methods. Furthermore, the confirmatory GC–MS method established in this study achieved better sensitivity and reduced analytical time and cost by eliminating the derivatization step and shortening the chromatographic run time.

2. Experimental

2.1. Chemicals and reagents

K, ketamine-d_4 (K-d_4), norketamine (NK), norketamine-d_4 (NK-d_4) were purchased from Cerilliant (Austin, TX, USA). Dehydronorketamine (DHNK, >99.5% purity) was synthesized by the Department of Chemistry, National Chung-Hsing University (Taichung, Taiwan, ROC). HPLC-grade methanol was purchased from Mallinckrodt (Phillipsburg, NJ, USA). Ethyl acetate, ammonium hydroxide, sodium bicarbonate, and sodium carbonate were all reagent grade. Ultra-pure water was produced with a Milli-Q purification system from Millipore (Bedford, MA, USA). Oasis HLB solid-phase extraction (SPE) cartridges (3 CC, 60 mg) were purchased from Waters Corp. (Milford, Ma, USA). Ketamine ELISA kits were purchased from Neogen Corporation (Lexington, KY, USA).

Standard stock solutions of K, NK, and DHNK (100 μg/mL) were prepared in ethanol and stored at −20 °C. Working standard solutions (K, NK, DHNK) and internal standard solutions (K-d_4, NK-d_4) for calibration and quality control were prepared in ethanol (10 μg/mL in each) and stored at 4 °C. Sodium carbonate–sodium bicarbonate buffer (carbonate-bicarbonate buffer, pH 9.5) was prepared by dissolving 2 g sodium carbonate and 9 g sodium bicarbonate in 1 L ultra-pure water and kept at room temperature. The rinse solution (50:50:2, methanol/water/ammonium hydroxide) for SPE was prepared daily.

2.2. Test specimens, calibrators, controls, and sample preparation

Two hundred and six urine specimens were systematically sampled nationwide from suspected drug users submitted by law enforcement agencies during the summer of 2004.

The six calibrators contained 30, 60, 100, 400, 700, and 1000 ng/mL of K, NK, and DHNK, respectively, and 100 ng/mL of K-d_4 and NK-d_4. Quality controls contained 40, 200, and 900 ng/mL of the analytes as the low, medium, and high controls, respectively. They were prepared by separately adding designated amounts of the working standard solution into 1-mL drug-free urine.

K-d_4 and NK-d_4 were used as the internal standards for the quantitation of K and NK, respectively. NK-d_4 was also used as the internal standard for DHNK for lack of deuterated DHNK. Test specimens, calibrators, and controls were first spiked with 10-μL internal standard solution (equivalent to 100 ng/mL internal standards) and then alkalized with 1-mL carbonate–bicarbonate buffer and vortexed. Extraction was proceeded with Oasis HLB SPE cartridges on a Zymark automated solid-phase extraction system (Hopkinton, MA, USA). The cartridge was first conditioned with 2-mL methanol, followed by 2-mL ultra-pure water and then 2-mL carbonate–bicarbonate buffer. After loading the sample, the cartridge was washed with 2-mL rinse solution. Analytes retained were subsequently eluted with 2-mL methanol following a 0.1-min nitrogen purge. The methanol eluate was evaporated to dryness at 40 °C under a nitrogen stream and reconstituted with 100-μL ethyl acetate prior to GC–MS analysis.

2.3. GC–MS confirmation

An Agilent 6890 GC/5973 MSD system was adopted in this study using a HP-5MS column (30 m × 0.25 mm I.D., 0.25 μm film thickness). GC oven temperature was programmed to rise

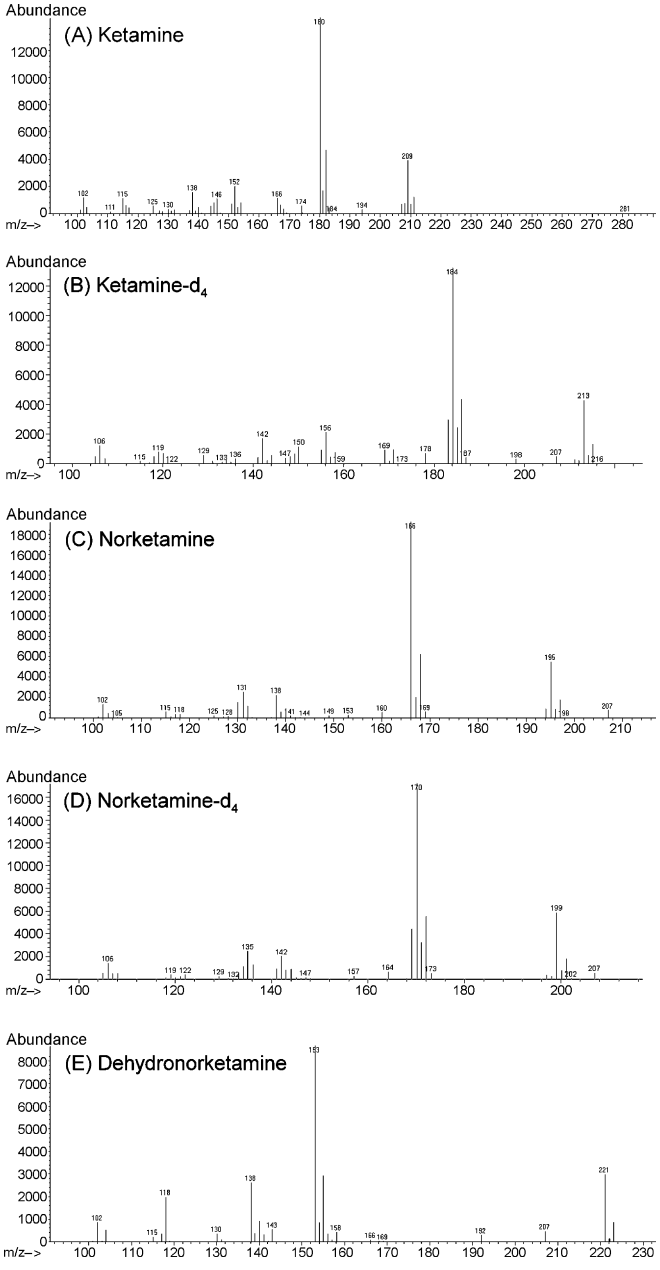

Fig. 1. Full scan mass spectra of K, K-d_4, NK, NK-d_4, and DHNK.

initially from 130 to 170 °C at 10 °C/min, to 200 °C at 5 °C/min, and then to 280 °C at 40 °C/min, and finally held at this temperature for 0.5 min. A 2-μL aliquot was injected in splitless mode. The injection port and the transfer line temperatures were set at 260 and 280 °C, respectively. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The MS was operated in electronic impact (EI) mode, and selected ion monitoring (SIM) mode was used for the identification and quantification of K, NK, and DHNK.

2.4. ELISA screening

Urine samples were tested following the Neogen's test procedure using an Anthos aw1 automatic microplate washer (Lagerhausstr, Wals, Austria). Twenty microliter aliquots of samples (test specimens, calibrators, and controls) were added to the appropriate wells in triplicate. One hundred and eighty microliter of diluted (1:180) drug–enzyme conjugate was added. The plate was covered, mixed by gentle shaking, and incubated at room temperature for 45 min. Each well was then washed five times with 300 μL diluted (1:10) wash buffer. One hundred fifty microliter of the K-Blue Substrate was then added, mixed by gentle shaking, and incubated at room temperature for 30 min. Fifty microliter Neogen's Red Stop Solution was added to stop enzyme reaction. The absorption was then measured at 650 nm using a Multiskan RC Microplate reader (Helsinki, Finland).

3. Results and discussion

3.1. Optimization and validation of GC–MS method

3.1.1. Ion selection for SIM mode

Full scan mass spectra of K, K-d_4, NK, NK-d_4, and DHNK were obtained (Fig. 1). The 5 most intense ions of each target compound were chosen for the cross-contribution evaluation under SIM mode. Table 1 shows the SIM response and cross-contribution of selected ions. Three ions with high relative abundance (>27%) and low cross-contribution were chosen for each target compound. All cross-contribution between target compounds and their deuterated internal standards were lower than 3% except *m/z* 182 for K but still acceptable (<5%). Retention time and ions selected for the five compounds for GC–MS confirmation are listed in Table 2. Two qualification ions were selected for each of the three target compounds, while one was selected for each of the two internal standards.

Fig. 2 shows the total ion chromatogram derived from drug-free urine spiked with 30 ng/mL of each analyte. Earlier GC–MS studies [9,16,18] reported the quantitation of K and DHNK without derivatization but failed to mention if these two analytes were well resolved. We noted a 60% cross-contribution of *m/z* 138 ion from K to DHNK when the same concentration of DHNK and K co-elute. In this study, the GC temperature was programmed to increase from 170 to 200 °C at a slow rate of 5 °C/min to adequately resolve DHNK (9.48 min) and K (9.63 min) peaks. The total run time was 12.5 min, which is faster than previously published methods [9,17]. Compared to derivatization with TFAA

Table 2
Retention time and ions selected for GC–MS confirmation and quantitation

Compounds	Retention time (min)	Selected ions (relative abundance, %)
Ketamine	9.63	180[a] (100.0), 182 (32.3), 209 (28.5)
Ketamine-d_4	9.59	184[a] (100.0), 213 (30.7)
Norketamine	9.09	166[a] (100.0), 168 (32.1), 195 (27.3)
Norketamine-d_4	9.06	170[a] (100.0), 199 (32.2)
Dehydronorketamine	9.48	153[a] (100.0), 138 (29.4), 221 (36.5)

[a] Quantitation ion.

Table 1
Peak area integration and cross-contribution data of ions selected for the analytes and internal standards by SIM mode

m/z	K	K-d_4	DHNK	*m/z*	NK	NK-d_4
Selected Ions for K				***Selected Ions for NK***		
138	101947	1092 (**1.1 %**)	-	**131**	182808	16545 (**9.1 %**)
152	130304	5770 (**4.4 %**)	-	**138**	154127	1184 (**0.8 %**)
180	925744	4812 (**0.5 %**)	-	**166**	1424809	4271 (**0.3 %**)
182	299283	12736 (**4.3 %**)	-	**168**	457683	13540 (**3.0 %**)
209	263560	2647 (**1.0 %**)	-	**195**	389459	1507 (**0.4 %**)
Selected Ions for K-d_4				***Selected Ions for NK-d_4***		
183	34510 (**17.5 %**)	197538	1710 (**0.9 %**)	**169**	47252 (**14.8 %**)	319940
184	9484 (**1.1 %**)	863986	2516 (**0.3 %**)	**170**	7731 (**0.6 %**)	1253529
185	2228 (**1.4 %**)	160980	1366 (**0.8 %**)	**171**	6069 (**2.7 %**)	228860
186	2693 (**1.0 %**)	280542	6465 (**2.3 %**)	**172**	4318 (**1.1 %**)	405039
213	1817 (**0.7 %**)	265405	1065 (**0.4 %**)	**199**	1642 (**0.4 %**)	403644
Selected Ions for DHNK						
118	-	28921 (**21.2 %**)	136431			
138	-	267 (**0.2 %**)	170168			
153	-	3700 (**0.6 %**)	578303			
155	-	50168 (**25.4 %**)	197550			
221	-	3738 (**1.8 %**)	211024			

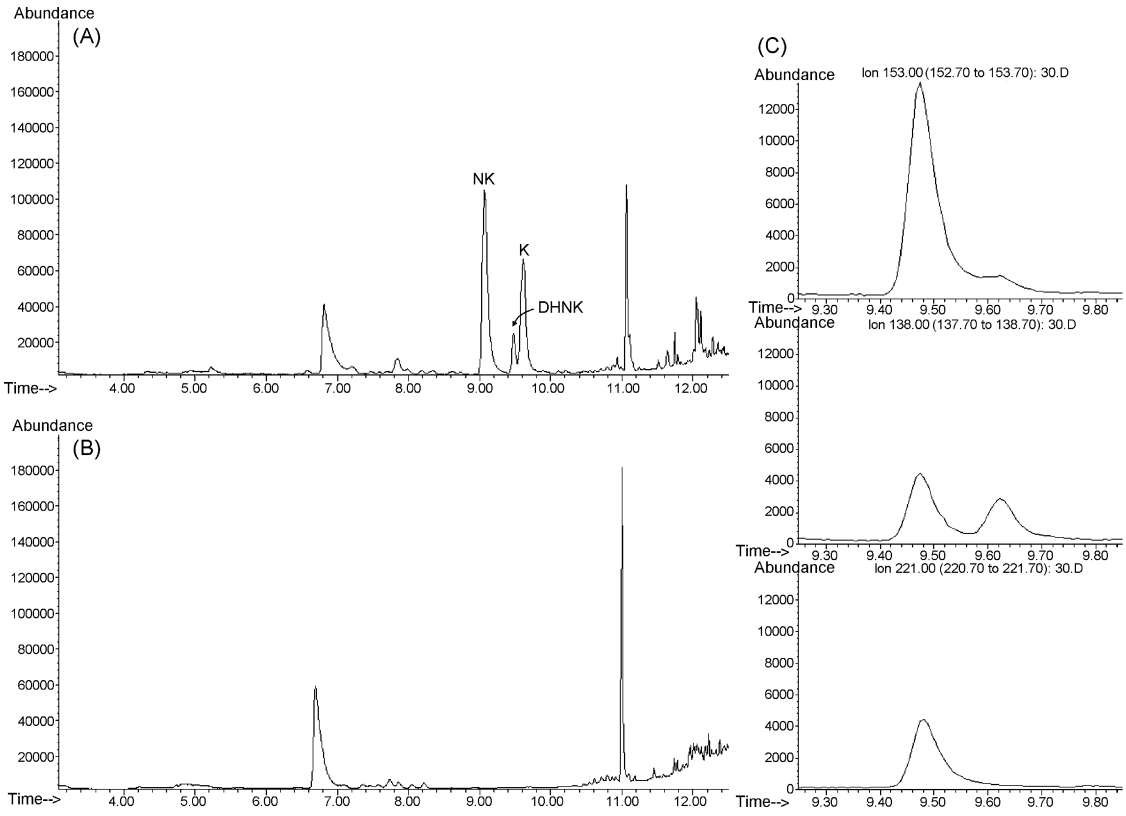

Fig. 2. (A) Total ion chromatogram of 30 ng/mL K, NK, and DHNK spiked urine. (B) Total ion chromatogram of blank urine. (C) DHNK selected ion chromatogram (*m*/*z* 153, 138, 221).

[6,10], HFBA [7], or MBTFA [17] prior to GC–MS analysis, this method is simple, fast, and more cost effective.

3.1.2. Linearity, LOD and LOQ

Calibration curves within the 30–1000 ng/mL range were linear for K, NK, and DHNK ($R^2 = 1.000$, 1.000, and 0.999, respectively). LOD was defined as the lowest concentration at which all replicates had qualification ion ratios within ±20% of the expected value. LOD was determined by analyzing a series of spiked urine samples ($n = 3$) with decreasing analyte concentrations and was 10 ng/mL for K, NK, and DHNK. LOQ was determined using the same criteria as LOD, and the quantitative results must be within ±20% of the expected value. The LOQs for K, NK, and DHNK were 15, 10, and 20 ng/mL, respectively. These LOD and LOQ definitions were more critical than using multiple signal-to-noise ratio (S/N) as the limits, which was generally adopted by chromatographic methodology. Moreover, these demanding definitions make drug confirmation test results more reliable and assist with forensic judgments as well. Previously published urinary quantification methods using GC–MS achieved a 5 ng/mL LOD (S/N > 10) for K by C_{18} SPE [14]; 1 and 5 ng/mL LODs (S/N > 3) for K and NK by liquid–liquid extraction (LLE) [9]; 2, 1, and 6 ng/mL LODs (S/N > 3) for K, NK, and DHNK by trifluoroacetic anhydride derivatization and LLE [22]; and a 1.5 ng/mL LOQ (S/N > 10) for both K and NK by *N*-methyl-bis(trifluoroacetamide) derivatization and C_{18} SPE [17]. The lowest concentration of spiked urine samples in this study ever tested was 1 ng/mL, and the S/Ns of K, NK, and DHNK were 23.5, 15.3, and 4.6, respectively. The higher S/Ns obtained at very low analyte concentration (1 ng/mL) indicated this analytical approach is more sensitive to K, NK, and DHNK than the previously published methods.

3.1.3. Precision, accuracy, and recovery

Accuracy, precision, and recovery of K, NK, and DHNK are listed in Table 3. Intraday accuracy ranged between 92.8 and 98.8%. Intraday precision ranged between 0.9 and 6.0%. Interday accuracy ranged between 90.2 and 104.5%. Interday precision ranged between 1.7 and 8.1%. Average recovery for K and NK was over 69%, and DHNK was lower (>53%). Good accuracy, precision, and acceptable recovery were achieved in this method.

3.2. Evaluation of ELISA and GC–MS methods with clinical specimens

The linear ranges of Neogen ELISA kit for K and NK were reported as 0–20 ng/mL and 0–500 ng/mL, respectively [23]. The study of nonhuman primate urine by Negrusz et al. [15] concluded that K was easily detectable at 25 ng/mL, and extraction of urine samples before ELISA allowed significant extension of the detection period. In our study, triplicates of the test specimen, calibrator and control samples were analyzed by the ELISA kit. One deviated measurement was excluded and the remaining two measurements were averaged and adopted to reduce false-positive (FP) and false-negative (FN) results.

Table 3
Accuracy, precision, and recovery of GC–MS analysis

Concentration (ng/mL)	Intraday		Interday		Recovery (%)
	Accuracy[a] (%)	Precision[b] (%)	Accuracy (%)	Precision (%)	($n=5$)
Ketamine					
40	96.7 ± 5.7	3.0	91.9 ± 15.0	8.1	77.9
200	96.6 ± 11.2	5.8	104.5 ± 10.5	5.0	73.0
900	98.8 ± 1.8	0.9	97.5 ± 5.1	2.6	69.4
Norketamine					
40	97.0 ± 6.2	3.2	90.2 ± 14.2	7.9	73.9
200	98.7 ± 11.8	6.0	103.9 ± 15.5	7.4	73.8
900	98.6 ± 1.8	0.9	97.1 ± 5.2	2.7	69.2
Dehydronorketamine					
40	92.8 ± 7.2	3.9	93.7 ± 14.8	7.9	–
200	95.3 ± 4.6	2.4	101.5 ± 8.1	4.0	53.4
900	96.6 ± 2.4	1.2	97.3 ± 3.4	1.7	61.5

[a] Accuracy(%) $= \left(\frac{\bar{X}_n}{C} \pm \frac{2S}{C}\right) \times 100\%$. $\bar{X}_n$: average, n: number of times.
[b] Precision(%) $= \frac{S}{\bar{X}_n} \times 100\%$. S: standard deviation, C: spiked concentration.

According to the manufacturer, the I-50 (the drug concentration shows 50% less color activity than the zero standard) for K and NK is 10 and 200 ng/mL, respectively. Linear ranges were 7–30 ng/mL for K and 250–400 ng/mL for NK. Logarithm curve further extended the K quantification capability up to 80 ng/mL. But in most cases, the K linear range was much lower than the concentrations in K user's urine specimens. On the other hand, NK would be undetectable if the specimens were diluted before ELISA screening. Single-point calibrator was used, and three concentration levels of K (10, 15, and 20 ng/mL) were evaluated in this study. FN rates were found to increase from 9.1 to 18.2% for the 206 clinical specimens tested when the screening cutoff was increased from 10 to 20 ng/mL. Therefore, 10 ng/mL K was chosen as the screening cutoff for the ELISA kit.

Test results of the 24 positive urine specimens detected by ELISA and/or GC–MS are listed in Table 4. In order to identify most of the recent K users, urine specimen was considered positive by GC–MS if any of K, NK, or DHNK concentration was higher than its LOQ.

3.2.1. Performance characteristics of ELISA and GC–MS as the test methodology

Table 5 shows the performance [24] of ELISA screening versus GC–MS confirmation on the 206 urine samples. ELISA showed good results and had advantages of high efficiency (98.1%), high sensitivity (90.9%), high specificity (98.9%), and low FP rate (1.1%). The 1.6% FP rate and 0% FN rate using 10 ng/mL K as ELISA screening cutoff and 15 ng/mL K as GC–MS confirmation cutoff (NK and DHNK LOQs not included) were better than those reported by Tan et al. [23], perhaps due to triplicate ELISA assay adopted in this study. When K, NK, and DHNK LOQs were used as GC–MS cutoff, 22 urine specimens were found positive, yet only 20 were screened positive by ELISA to result in a 9.1% FN rate. For the two FN specimens, K was not detectable by GC–MS, and the sum of the concentrations of NK and DHNK was lower than 70 ng/mL.

From our study, the ELISA kit showed a low K LOD, and screening costs may be saved if only duplicate rather than triplicate samples were used. ELISA screening followed with GC–MS confirmation is a suitable screening-confirmation system for K in urine specimens. However, a small percentage of the specimens with no K and low concentration of NK and DHNK may be missed by the screening method.

3.3. Concentrations of K, NK, and DHNK in urine specimens

K, NK, or DHNK were detected by GC–MS in 22 of the 206 urine specimens, in which 18 specimens (82%) contained all three analytes simultaneously. However, their concentrations vary widely (Fig. 3). The concentration range of K was the narrowest (20–7196 ng/mL) with the lowest median

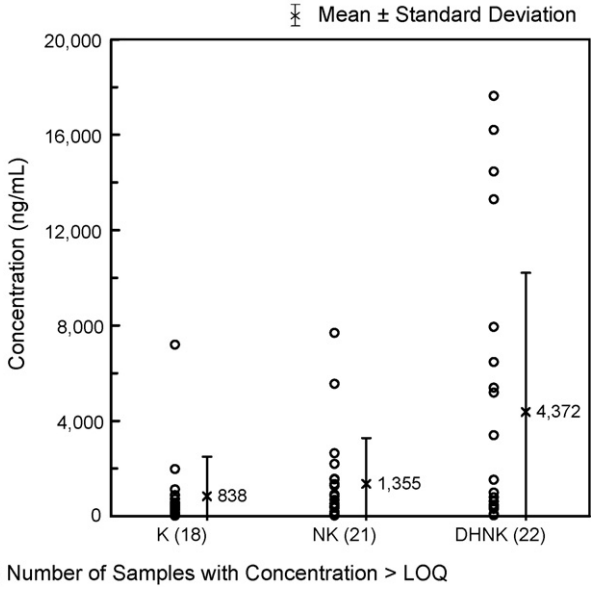

Fig. 3. Concentration of K, NK, and DHNK in positive urine samples.

Table 4
Test results of 24 K, NK or DHNK positive samples detected by ELISA and GC–MS

Sample no.	Screening test results	Confirmation test results			
		GC–MS[a]	Concentration (ng/mL)		
	ELISA		K	NK	DHNK
7–17	P	P	507	1542	17629
7–32	P	P	38	72	472
7–37	P	P	109	181	617
7–43	P	P	359	684	16,200
7–92	P	P	716	2188	5391
7–96	N	P	–	–	56
7–101	P	P	20	511	992
7–103	P	P	299	1272	5185
7–104	P	P	1976	1564	3392
7–109	P	P	155	415	367
7–110	P	P	71	49	90
7–113	P	P	7196	5548	465
7–114	P	P	304	841	1522
7–124	N	P	–	28	39
7–125	P	P	877	2644	13294
8–1	P	P	566	1350	14455
8–22	P	P	110	348	6464
8–24	P	P	–	25	36
8–40	P	P	262	502	477
8–49	P	P	1119	905	790
8–52	P	P	407	7685	7935
8–55	P	P	–	110	308
8–64	P	N	–	–	–
8–66	P	N	–	–	–

–, not detected or <LOQ.
[a] K, NK, or DHNK concentrations > LOQ (K 15 ng/mL, NK 10 ng/mL, DHNK 20 ng/mL).

(332 ng/mL), while the concentration range of DHNK was the widest (36–17629 ng/mL) with the highest median (891 ng/mL). The concentration range of NK was between 25 and 7685 ng/mL.

DHNK concentrations were generally higher than K and NK concentrations (20 in 22 cases), and 82% were over 100 ng/mL; NK concentrations were mostly in the middle (20 in 22 cases), and 77% were over 100 ng/mL; K concentrations were usually the lowest (17 in 22 cases), and 86% were under 1000 ng/mL. These findings were in agreement with the conclusions of Moore et al. [18] and Lin and Lua [9]. They have reported that the concentration of DHNK was greater than that of K and NK in most positive samples. No consistency of ratios between K, NK, and DHNK was found in these specimens, similar as reported by other studies [10,18].

Wieber et al. [25] reported that the urinary $t_{1/2}$ of K was 3.37 ± 0.14 h (undetectable after 22 h), $t_{1/2}$ of NK was 4.21 ± 0.35 h (undetectable after 22 h), and $t_{1/2}$ of DHNK was 7.21 ± 1.39 h (undetectable after 60 h). Thus, the two samples (ID 7–113, 8–49) having K concentrations greater than NK and DHNK in our study were probably collected soon after use.

3.3.1. DHNK as a biometabolite of K

In the early 1980s, DHNK was considered an artifact due to high-temperature GC procedures [8] or non-enzymatic dehydrogenation of the K metabolites [7]. Savchuk et al. [10] found that the ratios between K and its metabolites presented in urine from different patients varied considerably, and DHNK was identified as the major biotransformation product of the anesthetic

Table 5
Screening results and performance of ELISA

	Test results			Performance				
	Screening	GC–MS		Efficiency[a] (%)	Sensitivity[b] (%)	Specificity[c] (%)	FP rate[d] (%)	FN rate[e] (%)
		Positive	Negative					
ELISA	Positive	20	2	98.1	90.9	98.9	1.1	9.1
	Negative	2	182					

[a] Efficiency = (TP + TN) × 100/Total.
[b] Sensitivity = TP × 100/(TP + FN).
[c] Specificity = TN × 100/(TN + FP).
[d] False-positive rate = FP × 100/(FP + TN).
[e] False-negative rate = FN × 100/(TP + FN).

K. Subsequent studies based on LC–MS [18,19], conjugation studies [6,9], and K derivatization [17] all showed the presence of DHNK in biological samples. Since we found that DHNK concentrations were generally higher than those of K and NK, DHNK concentration range was the widest in real urine specimens. Also, internal standards K-d_4 and NK-d_4 analyzed individually by GC–MS showed no DHNK analogue. Thus, DHNK could not be a thermo-degradation artifact or non-enzymatic dehydrogenation product but a biometabolite of K.

4. Conclusions

We have developed a fast, sensitive, simple, and reliable GC–MS confirmation method for the determination of K, NK, and DHNK in urine. Good linearity, LOD, LOQ, accuracy, and precision were validated. Two hundred and six clinical urine specimens collected from suspected drug users were used to evaluate the screening efficiency of ELISA with the validated GC–MS protocol as the confirmation method. ELISA was shown to have high efficiency, high sensitivity, high specificity, low FP rate, and low FN rate when using 10 ng/mL K as cutoff. Combination of ELISA and GC–MS appeared to be a favorable two-stage test strategy for the determination of K in urine specimens. This system is suitable for high-volume routine urine tests and may be applied to other biological samples. This study also found that K, NK, and DHNK are simultaneously present in most positive specimens, and DHNK is a real metabolite of K in human urine, often with the highest concentration.

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