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Quantitative detection of ketamine, norketamine, and dehydronorketamine in urine using chemical derivatization followed by gas chromatography–mass spectrometry

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Abstract

A repeatable and highly sensitive analytical method using gas chromatography–mass spectrometry (GC–MS) in the selected ion monitoring mode (SIM) is developed for the simultaneous detection of ketamine (KT), norketamine (NK), and newly introduced dehydronorketamine (DHNK) in urine. The test specimen along with the deuterium analogues as internal standards (IS): d_4-KT for KT and d_4-NK for NK/DHNK, was extracted on an automatic solid-phase extraction (SPE) apparatus. The extracted eluate then was dried and derivatized with *N*-methyl-bis(trifluoroacetamide) ($CF_3CONCH_3COCF_3$, MBTFA). Finally, the cooled derivatized solution was directly injected into the GC–MS system for analysis. The proposed process achieves high sensitivity for the detection of KT, NK, and DHNK. Correlation coefficients derived from typical calibration curves in the range of 20–2000 ng/mL are 1.000 for KT and NK, 0.999 for DHNK. The limits of detection (LODs) and limits of quantitation (LOQs) are 0.5–1.0 and 1.5–3.0, respectively. The overall method recoveries of KT, NK, and DHNK are 82.2–93.4. The intra- and inter-day run deviations are smaller than 5.0%. The analytical scheme was also applied to the determination of KT, NK, and DHNK in 20 KT suspected urine specimens, and the results reconfirm that DHNK is a main metabolite of KT.
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Keywords: Ketamine; Norketamine; Dehydronorketamine; GC–MS; MBTFA

1. Introduction

Ketamine (KT) is catalogued as a synthetic anesthetic for human and veterinary surgery [1], it induces sedation, immobility, and amnesia. The drug is often called a dissociative anesthetic because a trancelike and cataleptic state with amnesia occurs with adequate doses [2,3]. KT is also structurally and pharmacologically related to phencyclidine [4], and is capable of producing some hallucination effects similar to those produced by phencyclidine. It was initially abused by medical personnel for its hallucinogenic effects, and gradually became popular on the European party scene in the early 1990s, then spread to other parts of the world. KT was stated to be metabolized to at least two major compounds of pharmacological interest: to norketamine (NK) by *N*-demethylation, which then is converted to dehydronorketamine (DHNK) by dehydrogenation [5], although some articles suggest that DHNK is an artifact resulting from the gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS) temperature programming process [6,7]. Analysis of KT and/or its demethylated metabolites has been accomplished by high-performance liquid chromatography [8–12] and liquid chromatography–mass spectrometry methods [13]. The analytes were also detected by gas chromatography with flame ionization detection [14,15], nitrogen phosphate detection [16], electron-capture detection [5], or by mass spectrometry detection with derivatization [6] or without derivatization [17–22]. Most of the above methods involved liquid–liquid extraction (LLE) or solid-phase

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extraction (SPE) for sample preparation; a method combining solid-phase microextraction and GC–MS has been reported, too [23]. KT is a seriously stereo-hindered secondary amine and is difficult to derivatize by most derivatizing reagents, so only one method using chemical derivatization (ChD) for KT urine testing was reported recently [6]. That article described the quantitative analysis of KT and NK using GC–MS preceded by LLE and pentafluorobenzoyl chloride (PFBC) derivatization. The current study explores a new and simplified approach in which the extraction is operated by automatic SPE equipment and the analytes are derivatized effectively by *N*-methyl-bis(trifluoroacetamide) ($CF_3CONCH_3COCF_3$, MBTFA). There have been some articles reporting that DHNK is an artifact of KT in the GC or GC–MS process [6,7]. Although some report said that DHNK is a metabolite of KT, yet it also suggested that DHNK may be an analytical artifact rather than a metabolite [24]. Because DHNK was not commercially available, almost none of the articles studied the quantitation of DHNK using GC–MS methods, and it is uncertain whether DHNK is a metabolite or an artifact. In this study, the protocol developed to simultaneously analyze KT, NK, and DHNK generates excellent recovery and assay linearity, and is applied to reconfirm that DHNK is an actual metabolite of KT when they are analyzed using GC–MS.

2. Experimental

2.1. Chemicals and reagents

KT, NK, d_4-KT, d_4-NK in methanol, supplied by Cerilliant (Austin, TX, USA), and DHNK (purity >99.5%) synthesized by National Chung-Hsing University (Taichung City, Taiwan, ROC) were diluted or dissolved to 10,000 ng/mL in alcohol as stock solutions. The solutions were then diluted to make the calibration standards and internal standards used in this study. These standards were refrigerated at 4 °C during the period of the study. MBTFA and the other derivatizing reagents were purchased from Supelco (Bellefonte, PA, USA). The SPE column, ISOLUTE HCX 130 mg, was supplied by International Sorbent Technology (Hengoed, Mid Glamorgan, UK). The derivatization vials were obtained from Supelco. Blank urine collected from drug-free volunteers was used to dilute the stock solutions to desired concentration standards.

2.2. Apparatus

Urine extraction was performed in automatic SPE equipment, a RapidTrace SPE workstation from Zymark (Hopkinton, MA, USA), Evaporation of the extracted solution by nitrogen purging was carried out in a Reacti-Therm apparatus from Pierce (Rockford, IL, USA). The GC–MS method was performed using an Agilent 6890 Series gas chromatograph connected with an Agilent 5973N Series mass selective detector (Wilmington, DE, USA). The instrument was operated in full-scan mode to search for the fragmentation pattern and in selected ion monitoring mode (SIM) to determine the qualifier and quantifier ions. The GC column used was an Agilent HP-5MS capillary column (30 m × 0.2 mm I.D., 0.25 μm film thickness). The GC was operated in splitless mode when performing injection with an Agilent 7673 autosampler. The injector temperature was 260 °C, interface temperature was 230 °C, column temperature was programmed from 90 to 280 °C at 15 °C/min, with the initial temperature held for 1 min and the final temperature for 3 min, total run time was 16.67 min; helium was used as the carrier gas at a flow rate of 1 mL/min.

2.3. Sample preparation

The test specimen (2.0 mL urine containing KT, NK, and DHNK) was placed in a disposable 10-mL glass test tube along with 100 μl d_4-KT stock solution as the internal standard for KT and 100 μl d_4-NK stock solution as the internal standard for NK and DHNK. The concentrations of KT, NK, and DHNK covered a range of 20–2000 ng/mL. Two milliliters of 0.1 M phosphate buffer at pH 6.0 was added to each of the test specimens and the glass test tube was then moved to the rack of the SPE equipment to carry out the automatic SPE process. At the beginning of the SPE procedure, the SPE column was conditioned with 2 mL methanol, followed by 2 mL deionized water and 2 mL of 0.1 M phosphate buffer at pH 6.0. The prepared specimen was loaded to the SPE column at a flow rate of 2.0 mL/min. The column then was washed with 1 mL of 1 M acetic acid and dried with nitrogen for 5 min. Finally, the analytes were eluted with 2 mL ethyl acetate containing 2% (v/v) concentrated ammonium hydroxide at 2.0 mL/min and collected in a 6-mL glass test tube. The collected eluate was then transferred to a derivatization vial and purged to dryness with nitrogen (2 kg/cm^2) at room temperature. One hundred microliters of MBTFA was added as ChD reagent to the vial, which was sealed tightly with a screw cap using a Teflon-surface silicone septum. The vial was shaken for 1–2 min and then heated at 120 °C for 20 min. The vial was then cooled to room temperature. One microliter of the derivatized solution was then directly injected into the GC–MS system.

3. Results and discussion

3.1. Sample preparation

3.1.1. Solid-phase extraction process

The SPE column is a mixed-mode cartridge in which two primary interactions are provided: both non-polar (C_8) and strong cation exchange (SO_3^-) functional groups. Methanol was initially added to condition the cartridge for activating the functional groups. The column was rinsed with phosphate buffer at pH 6 to keep the column weakly acidic; this keeps the C_8 and SO_3^- groups in free form and easy to bond with posi-

tive charged primary or secondary amine groups on KT, NK, or DHNK when the urine (buffered at pH 6) was eluted to the cartridge. Washing the bonded phase with water, solvent and acid, would selectively remove impurities or interferences, thereby increasing the recoveries of the analytes. Nitrogen was purged for column drying. Finally, eluting the analytes with ethyl acetate containing ammonia suppresses ionization of the basic drugs, counteracting both the ionic (SO_3^-) and non-polar (C_8) retention mechanisms, and allowing the analytes to be extracted effectively by the eluting solvent.

3.1.2. Selection of the derivatizing reagent

In this study, we have tried to use heptafluorobutyric anhydride (HFBA), pentafluoropropionic anhydride (PFPA), tri-

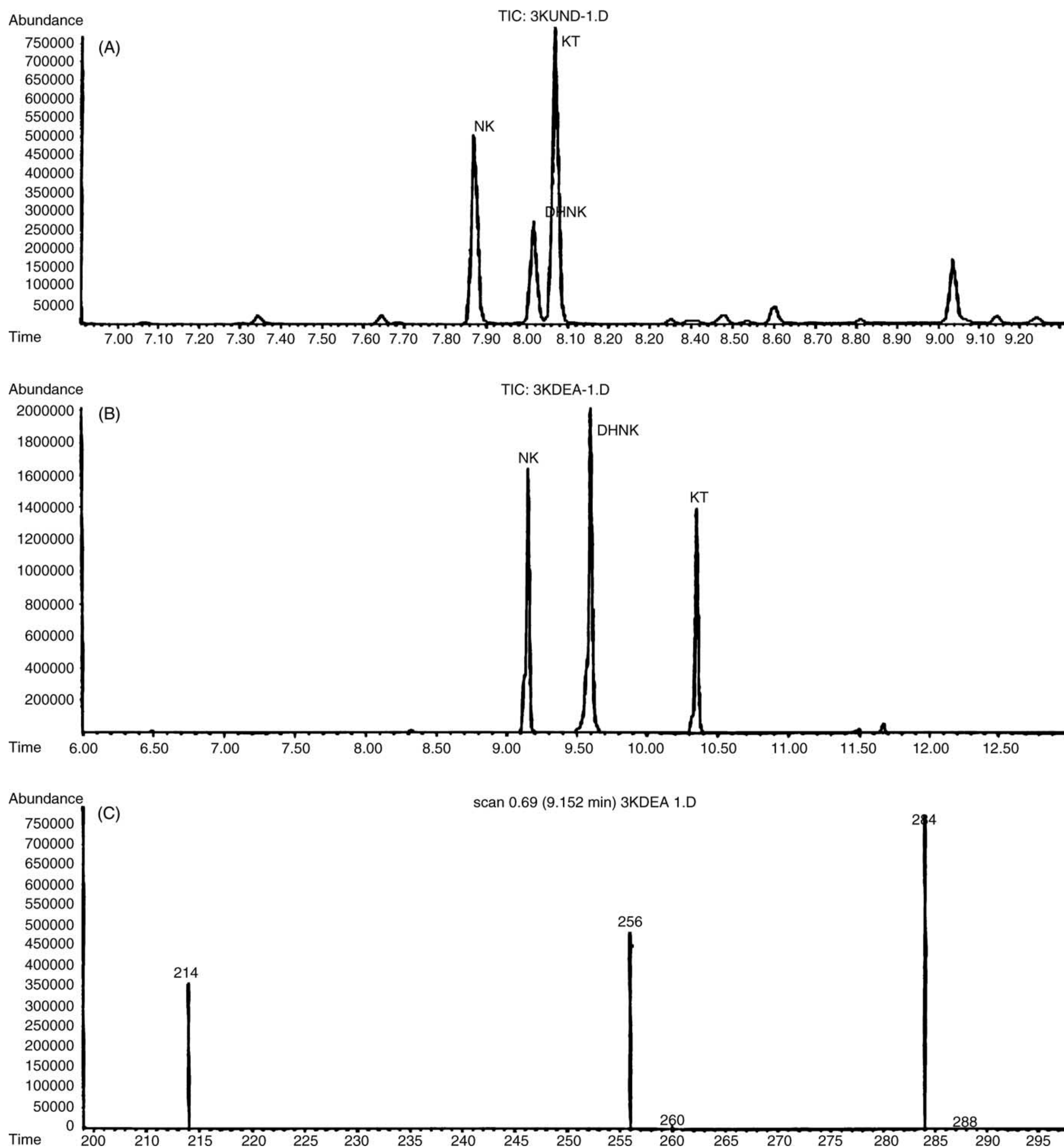

Fig. 1. (A) Total ion chromatogram of underivatized NK, DHNK, and KT. (B) Total ion chromatogram of derivatized NK, DHNK, and KT. (C–E) Mass spectra of derivatized NK, DHNK, and KT.

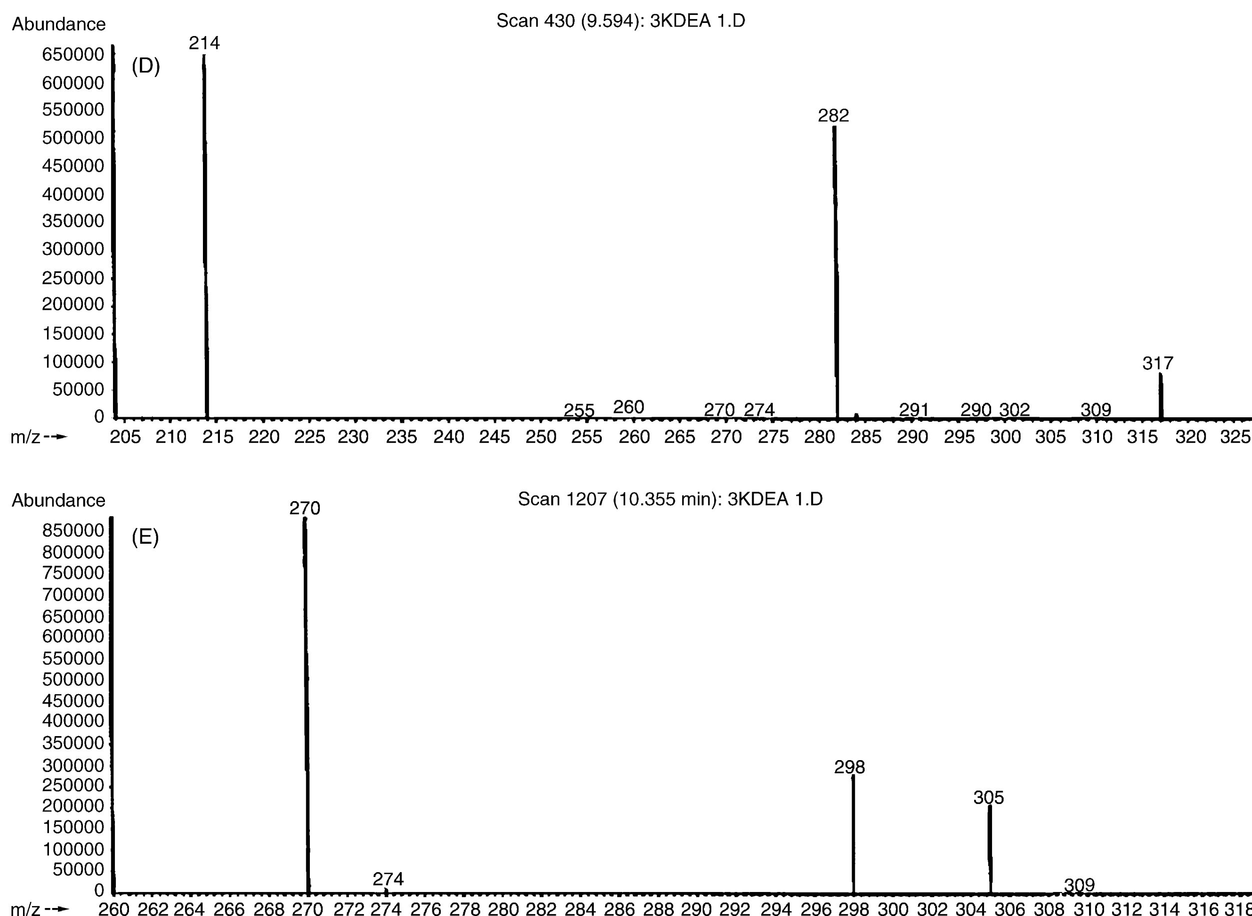

Fig. 1. (*Continued*).

fluoroacetic anhydride (TFA), and trichloroacetic anhydride (TCA) as ChD reagents to derivatize the three analytes at appropriate temperatures, most of the peak abundances of NK and DHNK were high enough for qualitating and quantitating, but the KT peak did not show satisfactory intensity under all of the conditions. We propose that, because the secondary amine on KT is stereo-hindered by the neighboring groups, the reaction between the derivatizing reagent and the N–H group is severely limited, resulting in low production of derivatized KT. Alternately, we also have tried to detect the three drugs without ChD. The monitored ions were chosen by running the unextracted stock solutions or from the reported articles [17]. The retention times and monitored ions of underivatized KT, NK, and DHNK are listed in Table 1. Fig. 1A shows the total ion chromatogram (TIC) of the three analytes. The instrumental response of KT in the chromatogram was high enough for identification and quantitation, but the KT and DHNK peaks interfere with each other and cannot meet the criteria for an acceptable chromatogram, which requires the peaks of interest be well resolved. Because ChD would produce the derivatized analytes with different volatility and electron affinity, ChD was expected to provide a better way to resolve the interference between KT and DHNK. We therefore used MBTFA as the derivatizing reagent to react with the analytes. Selection of monitored ions was carried out after acquisition of full-scan mass spectra for all analytes; qualifier ions were those at higher mass and of higher intensity, the ions used for quantitation were the qualifier ions having highest intensity. Retention times of the derivatized analytes and ions monitored (including internal standards) are listed in Table 2. Fig. 1B–E indicate a typical TIC and mass spectra of TFA–NK, TFA–DHNK, and TFA–KT. The TIC shows that the retentions are separated completely and the three peaks are well resolved and shaped. The instrumental intensities of the three compounds are also sufficiently high to be capable of performing a valuable method validation for KT urinalysis.

Table 1
The retention times (RTs), selected qualifier and quantifier ions (*m*/*z*) obtained upon the GC–MS analyses of underivatized KT analytes

Analyte	RT (min)	Qualifier ions	Quantifier ion
NK	7.869	138, 166, 195	166
DHNK	8.016	138, 153, 221	153
KT	8.068	152, 180, 209	180

3.1.3. Optimization of chemical derivatization

Optimal ChD conditions were determined by evaluating different heating temperatures and ChD times. Temperatures

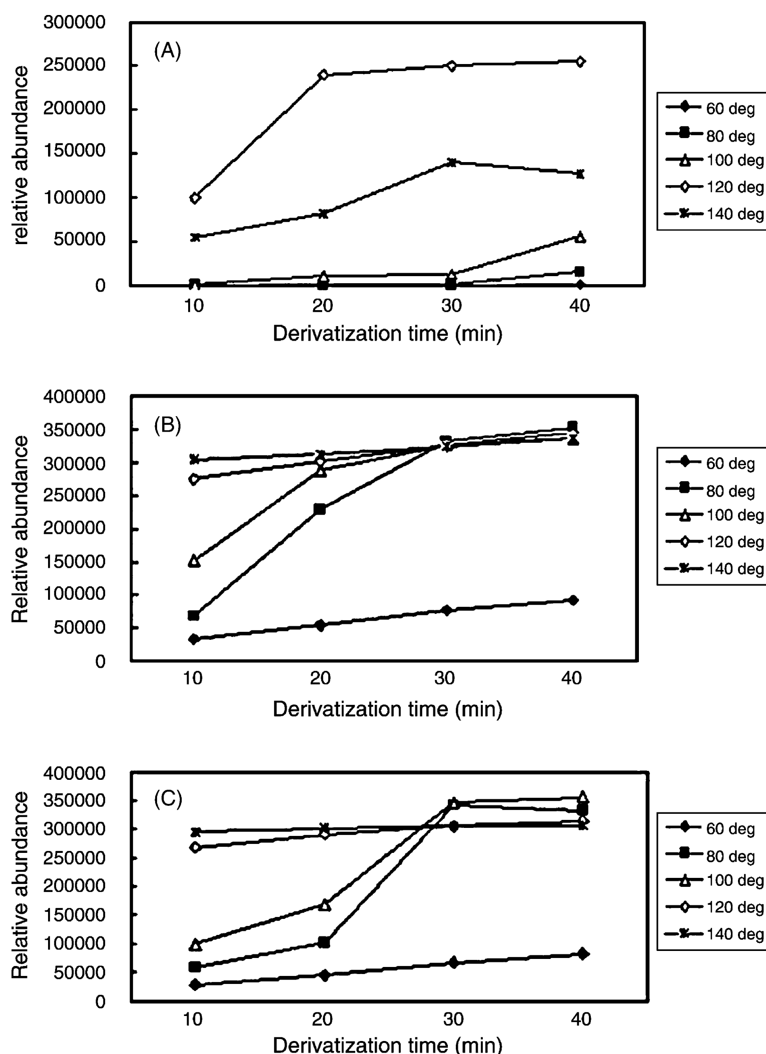

Fig. 2. The effect of derivatization time of KT (A), NK (B), and DHNK (C).

studied were 60, 80, 100, 120, and 140 °C, while the four ChD times studied were 10, 20, 30, and 40 min. The data shown in Fig. 2 indicates that for KT, the instrumental response appears to reach a maximum at approximately 20 min and 120 °C, while the maximum yield of derivatized KT does not occur at 140 °C. For NK and DHNK, the derivatizations are approximately finished at 10 min and 100 °C or at 30 min and 80 °C, respectively; and the yields of KT, NK, and DHNK are high enough to perform a method validation with high sensitivity and low detection limit. Based on these data, a combination of a 20 min derivatization time and a derivatization temperature of 120 °C is considered optimal and was used for the remainder of this study.

Table 2
The retention times (RTs), selected qualifier and quantifier ions (*m*/*z*) obtained upon the GC–MS analyses of MBTFA derivatized KT analytes and ISs

Analyte	RT (min)	Qualifier ions	Quantifier ion
TFA–d_4-NK	9.092	260, 288	288
TFA–NK	9.139	214, 256, 284	284
TFA–DHNK	9.594	214, 282, 317	214
TFA–d_4-KT	10.308	274, 309	274
TFA–KT	10.353	270, 298, 305	270

3.2. Detection of DHNK

To ensure that DHNK does not interfere with the identification and quantitation of KT and NK, an unextracted 2000 ng/mL DHNK standard was reacted with MBTFA, and then examined to see if there was any peak at the retention time of KT and NK in TIC. Fig. 3A demonstrates that no interfering peak is found; it indicates that DHNK will not affect the detection of KT and NK, and is suitable for use as one of the standards in urinalysis for KT.

To ascertain whether DHNK is an artifact produced from KT and NK in the GC/GC–MS process or is a metabolite of KT, 2000 ng/mL KT and NK standards were reacted with MBTFA, and tested to see if there was any peak at the DHNK retention time. Fig. 3B shows a typical chromatogram of KT and NK. Only the peaks of KT and NK are seen; there is no

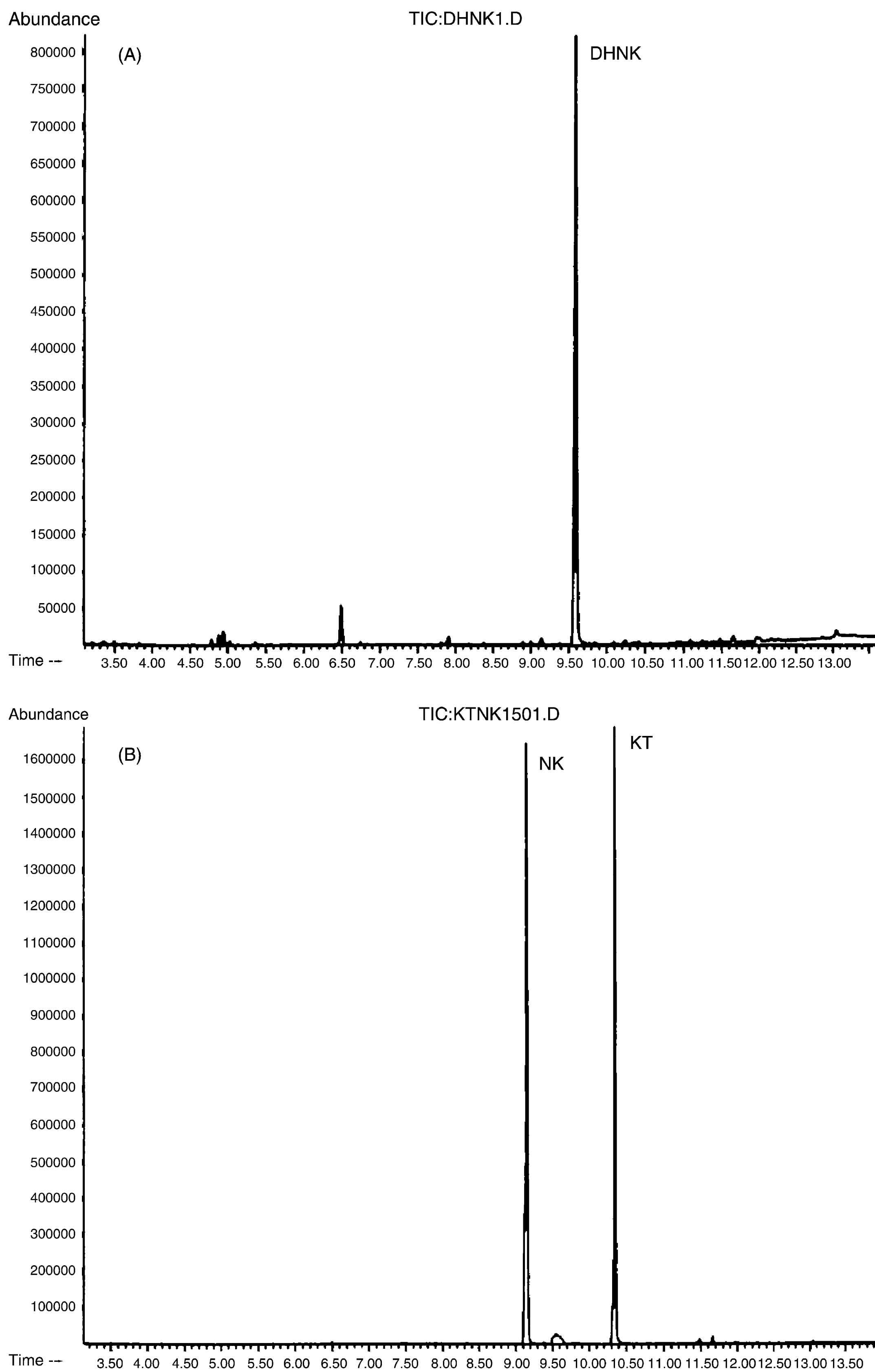

Fig. 3. (A) Total ion chromatogram of derivatized DHNK. (B) Total ion chromatogram of derivatized KT and NK. (C) Mass spectrum at the retention time of DHNK in (B).

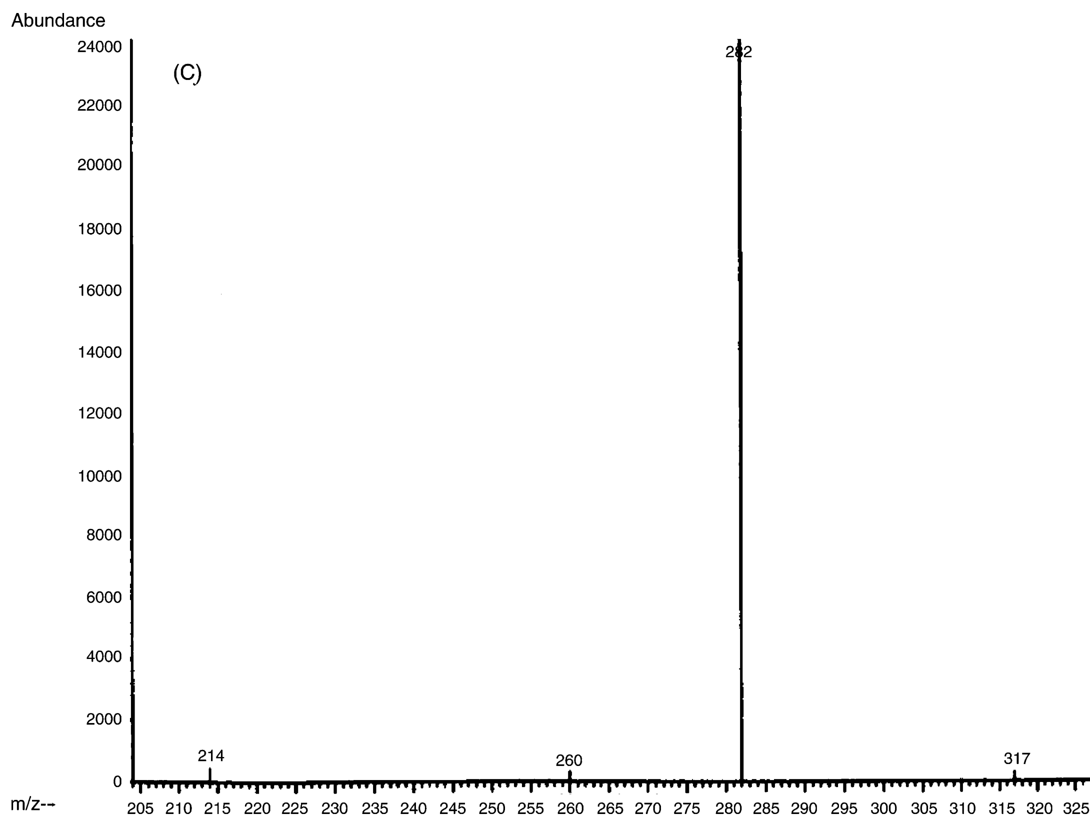

Fig. 3. (*Continued*).

peak found at the retention time of DHNK in Fig. 3C. This proves that DHNK is not an artifact of KT (or NK) resulting from the GC or GC–MS temperature programming process, DHNK is a bona fide metabolite of KT.

3.3. Linearity, limit of detection and limit of quantitation

To determine the linear ranges of the three compounds in the protocol, seven different concentrations of KT, NK, and DHNK-containing urine standards were prepared from the stock solutions. Calibration curves were based on plotting the KT/d_4-KT, NK/d_4-NK, or DHNK/d_4-NK peak area ratios against the concentrations of KT, NK, or DHNK, The correlation coefficients are 1.000, 1.000, and 0.999 for KT, NK, and DHNK.

The limit of detection (LOD) and limit of quantitation (LOQ) are based on the analyte concentrations giving peaks at the retention time in TIC with areas equal to the mean value of five blanks, plus the standard deviation of the mean value multiplied by 3 and 10. The LODs (0.5 and 0.5 ng/mL) of KT and NK, respectively, are lower than the previous reported values (4 and 3 ng/mL) [6]. Fig. 4A shows the TIC of derivatized NK, DHNK, and KT at 1.5 ng/mL, Fig. 4B shows the TIC of derivatized blank urine. The LOQs (1.5 and 1.5 ng/mL) are also much lower than those (13 and 7 ng/mL) of that article. The lower limits found in the present study indicate that acylation of KT and NK with MBTFA is more effective than acylation with PFBC.

3.4. Absolute recovery and reproducibility

Absolute recovery data were calculated by comparing the peak area of the standards processed through the entire SPE procedure against those derived from unextracted standard solutions in alcohol. Six replicates were used for calculating the intra- and inter-day reproducibility studies. The absolute recoveries of KT and NK are 82.2–93.4% and are higher than all the values previously reported in the literature [6]. All of the relative standard deviations for intra- and inter-day analysis are lower than 5.0%.

3.5. Case study

The protocol proposed in this study provides a good choice for KT confirmatory testing. We analyzed 20 KT suspected urine specimens from police arrestees by use of the analytical scheme described above; the relevant data are shown in Table 3. These data indicate: (a) samples 11 and 12 are NK and DHNK positive; (b) samples 18 and 20 are KT and NK positive only and no DHNK is detected, the results once again demonstrated that DHNK is not an artifact in the GC temperature programming process; (c) the KT concentrations

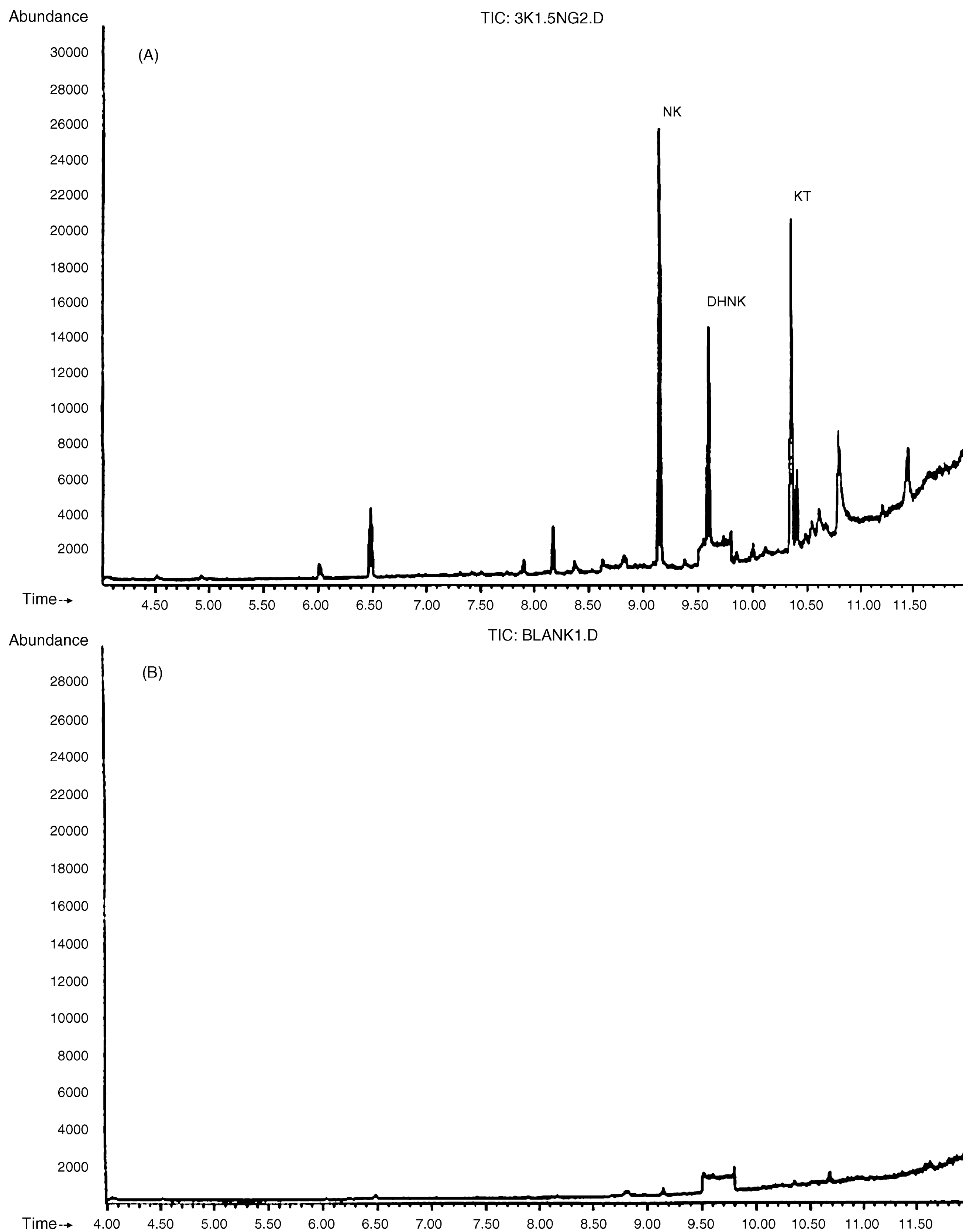

Fig. 4. (A) Total ion chromatogram of derivatized NK, DHNK, and KT at 1.5 ng/mL. (B) Total ion chromatogram of a derivatized blank urine.

Table 3
Concentrations obtained upon the total analyses of 20 suspected urine specimens (ng/mL)

No. of sample	KT	NK	DHNK
1	644	1885	9542[a]
2	1193	1189	1453
3	2371	1352	862
4	129	613	1421
5	523	1457	4408
6	86	840	13368
7	6515	9812	51268
8	2386	11490	63867
9	955	7225	79974
10	297	1624	12368
11	–[b]	227	574
12	–	96	209
13	1017	660	417
14	718	295	190
15	1207	2176	4997
16	175	2005	12399
17	434	484	78
18	1725	1010	–
19	634	291	57
20	460	570	–

[a] The sample is diluted to 10–50 times when the concentration is higher than 2000 ng/mL.
[b] The concentration is lower than 50 ng/mL.

are lower than 3000 ng/mL except for sample 7; and (d) the DHNK concentrations in samples 1, 5–10, 15, and 16 are much higher than the concentrations of KT or NK. These facts also demonstrate that DHNK is a genuine main metabolite of KT or NK, and should be used as a target compound in KT urinalysis as Moore et al. [13] found.

4. Conclusions

The results presented in this study indicate that automatic SPE and ChD using MBTFA followed by SIM detection of GC–MS is an effective analytical process to determine the concentrations of KT, NK, and DHNK in urine specimen. Because the peaks of underivatized KT and DHNK in TIC overlap each other, ChD prior to GC–MS analysis is necessary for improving the resolution between these RTs and for enhancing the instrumental responses of these two compounds. However, the fact that the stereo-hindered KT is difficult to react with many derivatizing reagents makes the development of ChD methods more difficult. Fortunately, the scheme presented here to simultaneously derivatize KT, NK, and DHNK allows their quantitative analysis with good reproducibility and recovery, and with low quantitation limits. On comparison with work reported recently [16], one sees that the trifluoroacetyl group of MBTFA used in this research is much smaller than the pentafluorobenzoyl group of PFBC in that reference, which results in producing a better yield of acylated KT in a shorter derivatization time (20 min versus 1 h). The derivatized solution can be injected into the GC–MS system directly, eliminating the drying and reconstitution steps of the acylated solution in the sample preparation procedure. The proposed protocol allows a fast, sensitive, automatic and high yield analysis for determining KT, NK, and DHNK. It is highly applicable to routine urinalysis and other biological sample testing. The case study demonstrates that this method is able to detect and quantify KT, NK, and DHNK in real samples. The results also show that DHNK is a major metabolite of KT.

Acknowledgments

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