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Gas chromatography-isotope dilution mass spectrometry preceded by liquid–liquid extraction and chemical derivatization for the determination of ketamine and norketamine in urine

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Abstract

An analytical scheme using gas chromatography (GC)-isotope dilution mass spectrometry (MS) assisted by precedent liquid–liquid extraction (LLE) and chemical derivatization (ChD) is described for the simultaneous determination of ketamine (KT) and its major metabolite, norketamine (NK), in urine. The simultaneous ChD of the two analytes, one primary amine and one secondary amine, with pentafluorobenzoyl chloride (PFBC) has not only enhanced their instrumental responses and mass-spectrum uniqueness but also afforded more proper yet easier selection of qualifier and quantifier ions and hence achieved more evidential identification and quantitation. Thus, the regression calibration curves for KT and NK in urine are linear within 100–5000 ng/ml, with correlation coefficients typically exceeding 0.99 and NK curves exclusively showing larger slopes than KT curves. The method limits of detection (LODs) determined by two definitions for KT and NK range from 3 to 75 ng/ml, and limits of quantitation (LOQs) from 9 to 100 ng/ml. The mean recoveries ($N = 3$) calculated for the LLE/ChD of KT and NK from 50 and 100 μl, respectively, of a 100 μg/ml urinary spike vary from 71.0 to 97.8%, with NK consistently giving better recoveries than KT. The precisions ($N = 3$) calculated for the total analyses of four real-case samples are typically below 12.3%. GC–MS operated in the positive ion chemical ionization (PCI) mode can offer both qualitative and quantitative information complementary to those given by the EI mode. The proposed scheme is simple, effective, reliable, and robust. It may serve as a confirmatory protocol for forensic urine drug testing.

Keywords: Derivatization, GC; Ketamine; Norketamine

1. Introduction

Ketamine (KT) is a synthetic, sedative, non-barbiturate, and fast-acting anesthetic commonly used during surgical procedures in both animals and humans. Dissociative and hallucinatory effects are also produced quickly by low doses of the drug. Between 1966 and 1970 more than 100 articles pertaining to KT anesthesia and its dissociative properties appeared in the worldwide scientific literature [1–3]. Articles on the use of KT in animals began appearing in about 1969 [4,5]. In the early 1970s, KT was diverted from legitimate uses to the illicit drug market by individuals who were seeking the above side effects seen in surgery patients during emergence from anesthesia, including vivid dreams, out-of-body experiences and delirium. Larger street doses are frequently used by the abuser in an attempt to yield "near-death" experiences [6]. Even higher does or poly-drug use can cause convulsions and death [7–9]. In 1995, KT was added to the DEAs emerging drugs list, and in 1999 became a Schedule II Controlled Substance in the States [10]. In Taiwan, there has recently also been a drastic increase in the abuse of this so-called "newly emerging drug of abuse".

KT is currently not one of the NIDA-5 standardly tested for in the basic drug test, nor is it included in the extended drug tests [11]. Theoretically, it is possible to test for the presence of KTs, i.e., KT and its major metabolite, norketamine (NK), as well as the somewhat controversial or presumptive metabolite, dehydronorketamine (DHNK) (Fig. 1) [12–16], in urine, blood, and hair, but in practice it is an

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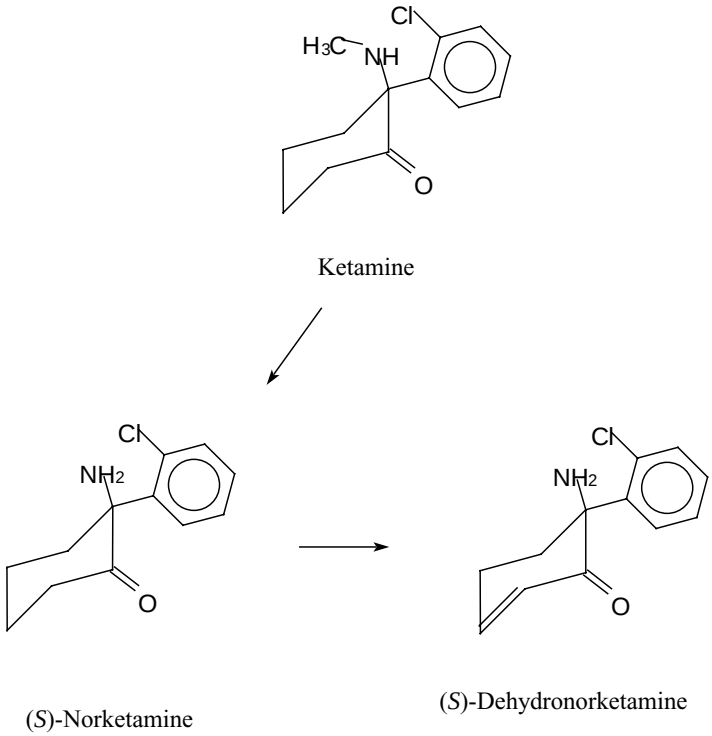

Fig. 1. Chemical structures of ketamine, (*S*)-norketamine and (*S*)-dehydronorketamine in the ketamine metabolism.

uncommon test to do. Unless there is a particular reason to look for them, as in the case of an autopsy, specific KTs tests are not normally conducted. In the past couple of years KT has become more widely known in the popular media and some testing companies have added it to their "Club Drug" testing battery. However, to the best of our knowledge, so far there have been no commercially available immunoassay kits purposely designed for KT detection. It has been claimed that KT may cause false positives for its analogue, phencyclidine (PCP), on some drug screens [11]. Consequently, a cautious screen for KTs relies on less specific or broader based procedures. A thorough literature search indicates that most laboratories have looked for KTs in their general "alkaline" extracts [17–22]. More recently solid-phase extraction [9,23,24] and solid-phase microextraction [25] have also been employed as sample preparation methods. As to the detection, most reports have been based upon thin-layer chromatography (including the commercially marketed "Toxi-Lab" system) [26–28], gas chromatography (GC) (with mass spectrometric (GC–MS [8,9,17,23,26,29]), flame ionization [8,27,30], electron capture [31–33] and/or nitrogen–phosphorus [20] detection), or liquid chromatography (with ultraviolet or photodiode array ultraviolet detection) [22–24,34,35]. However, only the minority of those reports deal with quantitative analysis [8,9,23,30–35], and even fewer go into chemical derivatization (ChD) [9,31–33]. The only ChD procedures ever described for KT were trifluoroacetylation [32] and heptafluorobutyrylation [31,33], both being for the GC assay [31–33]. The isotope dilution MS that has been extensively adopted in the workplace drug testing community appears only once for KTs analysis, with self-prepared NK-d_6 being the IS and the procedure somewhat simplified [36]. The previous unavailability of deuterium-labeled ISs seemed to have induced researchers to choose lignocaine [9] and 2-(*o*-bromophenyl)-2-methylaminocyclohexanone [31–33] as non-deuterium-labeled substitutes or simply conduct an external-standard calibration [29].

The criteria adopted by the US Federal Workplace Drug Testing Programs for conclusive drug identification and quantification require the appearance of the monitored ions at correct retention times (RTs) with acceptable intensity ratios among these ions. The RTs and ion intensity ratios observed in the test sample are compared with those established by an (or a set of) authentic calibration standard(s) incorporated in the same analytical batch [37]. It is well known that usually these criteria can be best met by utilizing isotope dilution MS with the analyte properly derivatized. Since KT-d_4 and NK-d_4 have become commercially available and our laboratory has long been engaged in isotope dilution MS analysis of drugs of abuse, we would like to conduct a detailed evaluation in an effort to develop a quality confirmatory protocol for forensic urine KTs testing.

2. Experimental

2.1. Materials

Racemic *d,l*-ketamine hydrochloride (KT·HCl; 1 mg/ml in methanol), *d,l*-norketamine hydrochloride (NK·HCl;

1 mg/ml in methanol), *d,l*-ketamine-d_4 hydrochloride (KT-d_4·HCl; 0.1 mg/ml in methanol) and *d,l*-norketamine-d_4 (NK-d_4·HCl; 0.1 mg/ml in methanol) were purchased from Cerilliant Co., USA. Pentafluorobenzoyl chloride (PFBC) was from Sigma Chemical Co., USA. Ethyl acetate (EA), sodium carbonate, triethylamine (TEA), and cyclohexane were from Fisher Chemical, USA. Methane of 99.99% purity was obtained from a local gas supplier in Taipei. All of the above agents and solvents were in analytical or reagent grade and were directly used without further purification.

2.2. Sample preparation

2.2.1. ChD of authentic KT·HCl and NK·HCl with PFBC

For plotting the instrumental calibration curves, an authentic 100 μg/ml KT·HCl/NK·HCl binary solution in EA and a 100 μg/ml KT·HCl-d_4/NK·HCl-d_4 binary solution in EA as ISs were prepared. An appropriate amount (1, 5, 10, 20, 30, 40, 50 μl) of the authentic sample solution was added to a screw-cap topped derivatizing tube containing 20 μl of the ISs solution. A 100 μl portion of EA and 50 μl of PFBC were added. The mixture was incubated at 105 °C for 1 h, transferred to a concentration tube, and purged at 45 °C with nitrogen gas to dryness. More EA was added to make up a 200 μl solution. A 1 μl aliquot of this solution was injected for the GC–MS analysis.

The above resulting solution was also used in evaluating the efficacies of the ChD of KTs with PFBC. To prepare the free base (so as to be truly GC–MS compatible, i.e., total-amount vaporization in the injection port) of the underivatized control, an appropriate amount (1, 5, 10, 20, 30, 40, 50 μl) of authentic sample solution was added to a screw-cap topped derivatizing tube containing 20 μl of ISs solution. To this mixture were added 1 ml of sat. sodium carbonate solution and 4 ml of 3:1 (v/v) TEA/cyclohexane. After 10 min of mechanical shaking, the mixture was allowed to stand still. The upper layer was transferred to a concentration tube, and purged at 45 °C with nitrogen gas to dryness. More EA was added to make up a 200 μl solution. A 1 μl aliquot of this solution was injected for the GC–MS analysis.

2.2.2. Liquid–liquid extraction (LLE) and PFBC derivatization of KT·HCl/NK·HCl fortified and unknown urine specimens

For plotting the method calibration curves, an appropriate amount (1, 5, 10, 20, 30, 40, 50 μl) of the foregoing authentic sample solution was added to a screw-cap topped derivatizing tube containing 1 ml of blank urine and 20 μl of ISs solution. To each spike were added 1 ml of sat. sodium carbonate solution and 4 ml of 3:1 (v/v) TEA/cyclohexane. After 10 min of mechanical shaking, the mixture was subjected to centrifugalization at 3000 rpm for 5 min. The upper layer was transferred to another derivatizing tube. To the remaining lower layer was added another 4 ml of 3:1 (v/v) TEA/cyclohexane, and the extraction steps were repeated. The combined extracts in the derivatizing tube were purged at 45 °C with nitrogen gas to dryness. The residues were re-dissolved with 100 μl of EA and to the solution 50 μl of PFBC was added. The mixture was incubated at 105 °C for 1 h, and, if necessary, centrifuged for another 5 min. The supernatant was transferred to a concentration tube, and purged at 45 °C with nitrogen gas to dryness. More EA was added to make up a 200 μl solution. A 1 μl aliquot of this solution was injected for the GC–MS analysis.

The liquid–liquid extraction (LLE)–ChD procedure for unknown urine specimens was the same as that for spikes except that the authentic KT·HCl/NK·HCl solution needed not be added.

2.3. GC–MS

2.3.1. With electron impact ionization (GC–EIMS)

The GC–EIMS analyses were carried out using a Hewlett-Packard HP-5890 Series II gas chromatograph coupled to an HP-5971 Series mass selective detector (MSD). The instrument was first operated in full-scan mode to look into the fragmentation nature (with the aid of a software named "High Chem™ Mass Frontier Version 1.0") of the previously unexplored PFBC-derivatized analytes, and then in selected ion monitoring (SIM) mode to further evaluate the qualifier and quantifier ions and run the formal analysis. The GC column used was a HP-5 MS capillary column (30 m × 0.2 mm i.d., 0.33 μm film thickness). The GC was operated in the splitless mode (i.e., purge off) when performing injection with the aid of an HP-7673 autosampler, but 1 min later the purge valve was turned on. The injector temperature was 250 °C. The column temperature was programmed from 100 to 250 °C at 25 °C/min, with the initial temperature held for 1 min and final temperature 15 min. Helium was used as the carrier gas at a flow rate of 1 ml/min. Effluents from the GC column was transferred via a transfer line held at 280 °C to a 70 eV EI ionization source held at 180 °C.

In performing GC–EIMS SIM quantitation of KTs, the calibration curves were produced by plotting the peak-area ratio (analyte:IS) against the concentration of the appropriate analyte in the fortified samples. The peak-area ratio used was the mean of triplicate analyses.

2.3.2. With positive ion chemical ionization (GC–PCIMS)

The GC–PCIMS analyses were carried out using a Finnigan MAT GCQ instrument operated in the full-scan mode accompanied by extracted ion chromatograms (EICs). The operation conditions were the same as those for the foregoing HP GC–MSD instrument except that instead of using a 70 eV EI ionization source methane was used as reagent gas and that the GC temperature program had minor changes: 70 °C (1 min) to 150 °C at 30 °C/min, hold 150 °C for 1 min, 25 °C/min to 250 °C, hold 250 °C for 15 min. Qualitative information was offered by the RTs and PCI mass spectra of the analytes while quantitative information by the EICs.

3. Results and discussion

3.1. Efficacies of simultaneous ChD of KT and NK with PFBC

A formal definition of "confirmation" involves the testing of a second aliquot of the same biological sample using a different chemical principle. Screening of the underivatized sample by immunoassay and confirmation of the derivatized sample by GC–MS qualifies as a confirmation and has currently been adopted by most of the prevalent urine drug testing protocols except for the KTs counterpart. The objectives of ChD prior to GC–MS analysis usually include conforming to GC environment, improving separation, enhancing detection, and assist structural elucidation. Based on our previous experience in dealing with other drugs, it was anticipated that any commonly used perfluorinated ChD should more or less achieve all of the above four objectives either through the GC part or through the EIMS part or through both. Having tried three acylation ChD reagents, i.e., trifluoroacetic anhydride (TFA), pentafluoropropionic anhydride (PFPA) and pentafluorobenzoyl chloride, and three silylation ones, i.e., *N*,*O*-bis(trimethylsilyl)acetamide (BSA), *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), and *N*-methyl-*N*-(*t*-butyldimethylsilyl)triflouoroacetamide (MTBSTFA), we found it was PFBC that could not only simultaneously derivatize KT, KT-d_4, NK, and NK-d_4 but also consistently result in the strongest instrumental responses of the derivatives. Although KT and KT-d_4 are secondary amines with a methyl group directly bonded to and two bulky moieties (i.e., chlorophenyl group and cyclohexanone structure) located in the vicinity of the nitrogen atom, and the approaching of the nucleophilic nitrogen to the carbonyl carbon of PFBC is more hindered compared to the situation with NK and NK-d_4 which are primary amines, PFBC is not only electrophilic enough but also capable of forming more stable transition states or intermediates with NK and NK-d_4 benefiting by the stronger resonance-stablizing effect of the benzoyl group. Nevertheless, as will be shown below, the PFBC–ChD yields for NK and NK-d_4 were generally higher than those for KT and KT-d_4, although many more than eight equivalents of PFBC had been used. At this stage, five basic advantageous conclusions can already be drawn for the PFBC–ChD of KTs: (1) the operation of the PFBC–ChD procedure is simple and rapid. (2) All the GC–MS full-scan total ion current chromatograms (TICs) of the PFBC–ChD crude products obtained from the urinary spikes have low noises (a typical GC–EIMS TIC is shown in Fig. 2), implying after the PFBC–ChD reaction there exist only limited amounts of underivatized KTs and/or by-products. (3) As shown in Table 1, the RT differences between KT and KT-d_4 and between NK and NK-d_4 are at most 0.02 min, while those between PFB–KT and PFB–KT-d_4 and between PFB–NK and PFB–NK-d_4 are lengthened at least to 0.034 min and up to 0.069 min. Although the relevant peaks in the TICs still look unresolved superficially, it was those small improvements on the RT differences that further reduced the otherwise still noticeable "analyte-IS (ion) cross contributions" (said of the already well-selected qualifier and quantifier ions) to truly negligible levels (more details in Section 3.2). (4) Benefiting by their higher volatility and electron affinity, PFBC-derivatized analytes/ISs can yield more detectable ions. As indicated by the respective ordinates of tens of TICs, the overall (GC plus EIMS) instrumental responses of PFBC-derivatized analytes/ISs are typically 5–10 times

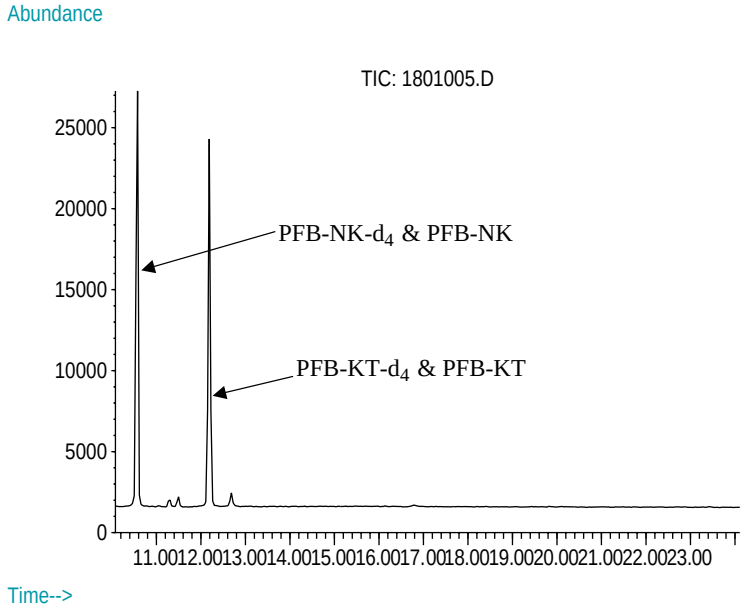

Fig. 2. A TIC chromatogram obtained upon the GC–EIMS full scan of a pretreated urinary spike containing PFB–NK-d_4 (left part of left peak), PFB–NK (right part of left peak), PFB–KT-d_4 (left part of right peak), and PFB–KT (right part of right peak).

Table 1
The retention times (RTs) obtained upon the GC–MS analyses of PFBC derivatized and underivatized KT anlytes and ISs

PFBC derivatized			Underivatized		
Analyte or IS	RT (min)		Analyte or IS	RT (min)	
	GC–EIMS	GC–PCIMS		GC–EIMS	GC–PCIMS
PFB–NK-d_4	11.346	17.41	NK-d_4	7.208	10.08
PFB–NK	11.380	17.46	NK	7.226	10.10
PFB–KT-d_4	13.209	20.84	KT-d_4	7.392	10.31
PFB–KT	13.278	20.89	KT	7.405	10.33

greater than those of the underivatized counterparts. (5) Taking GC–EIMS analysis as an example, the pure mass spectra of the four PFB–KTs (i.e., each spectrum obtained in a separate run upon an aliquot containing just one derivative) (Fig. 3) provide more structural information than those from the underivatized KTs [9,17,23,26]. Whereas only two or three ions are under consideration (in the contexts of their intensities and specificities) for being qualifier/quantifier ion "candidates" for each of the underivatized analytes (*m/z* 180 (base peak), 182, 209 for KT; *m/z* 166 (base peak), 168, 195 for NK) and they all generate essentially noticeable "analyte-IS cross contributions", it is easier to first pick up three to five candidate ions for each of the four PFB–KTs and then most appropriately select three "formal" qualifier ions, one of them serving as quantifier ion. Additionally, all the molecular ion peaks of the four PFB–KTs are more distinct than their underivatized counterparts. In sharp contrast to the close parallel between the fragmentations of KT/KT-d_4 and those of NK/NK-d_4, the fragmentation patterns of PFB–KT/PFB–KT-d_4 and PFB–NK/PFB–NK-d_4 are remarkably different. Nevertheless, except for *m/z* 382 of PFB–NK and *m/z* 386 of PFB–NK-d_4, which result from the loss of a chlorine atom, all other major ions are formed somehow initiated or accompanied by the cleavage of the C–C bond next to the nitrogen atom. By the way, it is also interesting that the non-hydrogen-bonded but heavier PFB–KTs are more retained in the low-polarity HP-5 capillary column than are the hydrogen-bonded underivatized KTs.

3.2. Selection of qualifier and quantifier ions

Judging from our previous experience in analyzing amphetamines, satisfactory GC resolution between KTs and their respective deuterium-labeled ISs may need the use of ISs labeled with more than eight or nine deuterium atoms [38]. Being more available, however, d_4-labeled ISs were used throughout the present study. This along with the demand of rapid analysis in practice did lead to inadequate GC separation between PFB–KT and PFB–KT-d_4 and between PFB–NK and PFB–NK-d_4 (Fig. 2). The appreciable overlap between the analyte peak and the IS peak caused their respective fragmented ions to be mutually mixed-up. Displayed in Fig. 4 are the analyte/IS cross-contaminated mass spectra of the two pairs of PFBC-derivatized analyte/ISs fetched from the two unresolved peaks in Fig. 2. The impure mass spectra of the analyte is to some extent similar to that of the IS. Fortunately, through the more appropriate yet easier selection of qualifier/quantifier ions, the essential or effective resolution (as opposed to the "superficial" resolution) and hence the accuracy and precision for the GC–EIMS and GC–PCIMS analyses can still be secured. Having gone through a detailed evaluation process in the light of "minimized analyte-IS cross contribution" according to that described by Liu for the quantitative determination of pentobarbital [37], the qualifier and quantifier ions decided for the GC–EIMS analyses of the four PFBC-derivatized KTs are listed in Table 2. It is encouraging that the "analyte-IS cross contributions" assessed for these qualifier and quantifier ions range from 0.55 to 3.5%, which are ideally small and really negligible. For instance, at first sight of the impure mass spectra in Fig. 4(a) and (b), it seems considerable proportion of PFB–KTs *m/z* 152 ions have been contributed by PFB–KT-d_4, and considerable proportion of PFB–KT-d_4s *m/z* 156 ions have been contributed by PFB–KT. However, more precise calculations of the pure mass spectra in Fig. 3(a) and (b) confirm that all but 1.75% (inevitably from PFB–KT-d_4) of the base-peak *m/z* 152 ions are from PFB–KT, and all but 1.60% (from PFB–KT) of the base-peak *m/z* 156 ions are from PFB–KT-d_4. Namely, *m/z* 152 is not only most sensitive to but also most specific for PFB–KT, and is therefore best suited for serving as PFB–KTs quantifier ion. Indeed, all of the strictly selected ions are in agreement with what were anticipated from the direct mass spectra interpretation. The inference structures of the respective qualifier and quantifier ions given by the "High Chem^{TM} Mass Frontier" are indicated in Fig. 3. To illustrate the practical value of the proposed isotope

Table 2
Qualifier and quantifier ions (*m/z*) selected for PFB–KT, PFB–KT-d_4, PFB–NK, and PFB–NK-d_4

Analyte or IS	GC–EIMS		GC–PCIMS	
	Qualifier ions	Quantifier ion	Qualifier ions	Quantifier ion
PFB–KT	152, 360, 368	152	432, 434	432 ($M+1$)
PFB–KT-d_4	156, 364, 372	156	436, 438	436 ($M+1$)
PFB–NK	312, 354, 382	354	418, 420	418 ($M+1$)
PFB–NK-d_4	316, 358, 386	358	422, 424	422 ($M+1$)

dilution GC–MS, Fig. 5 shows the reconstructed GC–EIMS SIM chromatograms resulting from the total analysis of a real-case sample.

So far as the selection of qualifier/quantifier ions is concerned, the GC–PCIMS mass spectra of the four PFB–KTs (Fig. 6) look trivial although PFB–KTs spectrum and PFB–NKs have also been contaminated by a few foreign ions from PFB–KT-d_4 and PFB–NK-d_4, respectively, and vice versa. For each PFB–KT, the two quasi-molecular ions, i.e., $m/z = M + 1$ and ^{37}Cl isotopic $M + 3$, best serve as qualifier ions, both being most characteristic and abundant ions of their own (Table 2). The $M+1$ ion is simultaneously best suited for the quantifier ion. The structures of the respective qualifier/quantifier ions are also indicated in Fig. 6.

3.3. Quantitation

The seven-point method calibration curves plotted for the GC–EIMS SIM anlyses of KT and NK in urine (equations: $y = 0.0062x + 0.0025$ for KT; $y = 0.0104x + 0.0031$ for NK) are both linear within 100–5000 ng/ml, with the correlation coefficients (r^2) being 0.995 and 0.999, respectively. Likewise, the seven-point method calibration curves plotted for the GC–PCIMS full-scan EIC analyses of KT and NK in urine (equations: $y = 0.0046x + 0.084$ for KT; $y = 0.0072x + 0.11$ for NK) are also both linear within 100–5000 ng/ml, with the correlation coefficients (r^2) being 0.993 and 0.998, respectively. Both of the two NK curves show larger slopes than the two KT curves do, suggesting that NK is easier to PFBC derivatize than KT.

3.4. Limits of detection (LODs) and limits of quantitation (LOQs)

The method limit of detection, (M)LOD, and method limit of quantitation, (M)LOQ, were determined in this study by two definitions. Definition A is currently more prevalent in the forensic practice [39]. After serial analyses of urinary

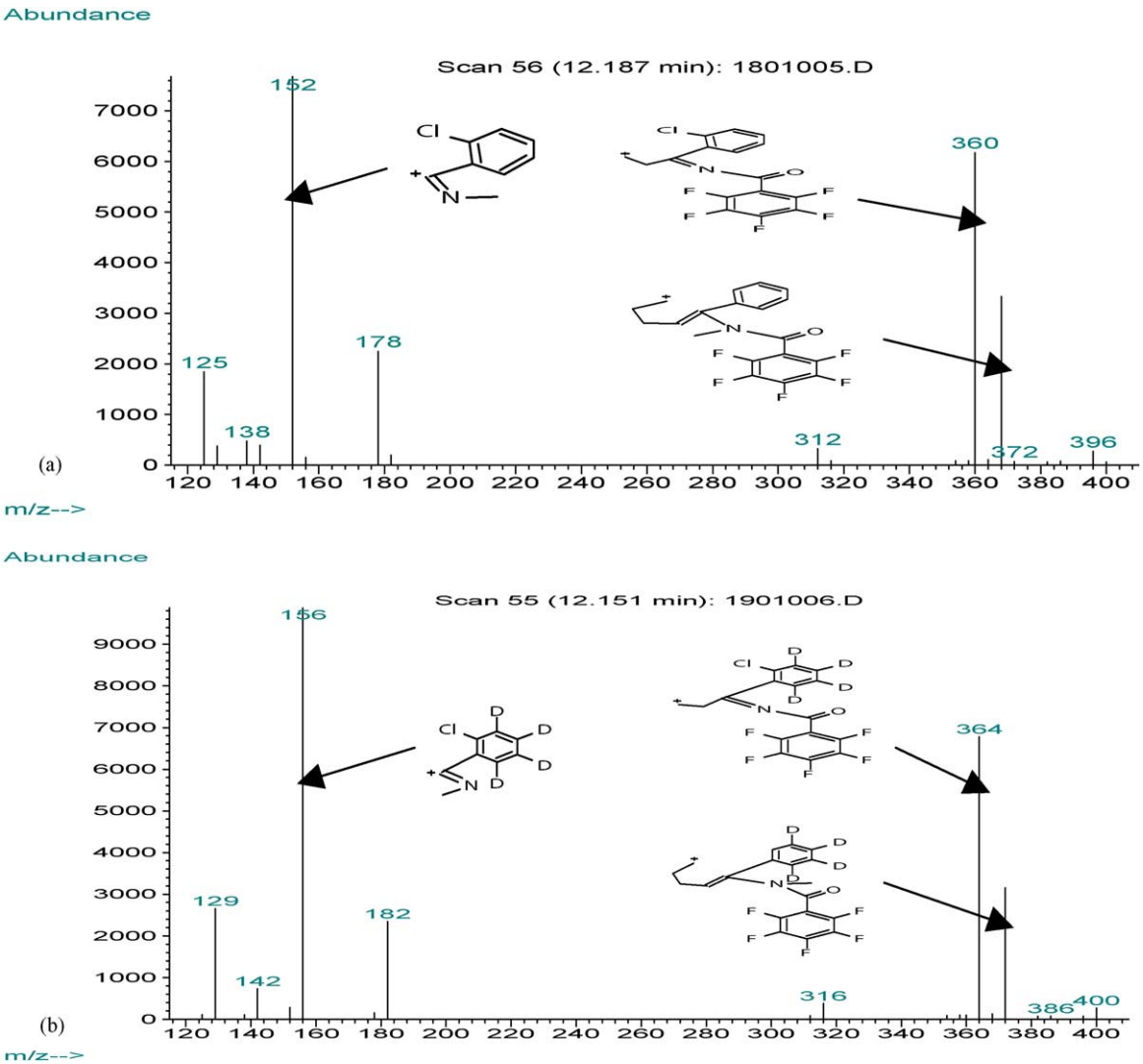

Fig. 3. The pure mass spectra of (a) PFB–KT, (b) PFB–KT-d_4, (c) PFB–NK and (d) PFB–NK-d_4 (i.e. each spectrum obtained in a separate run upon an aliquot containing just one compound). Only the key mass regions are shown.

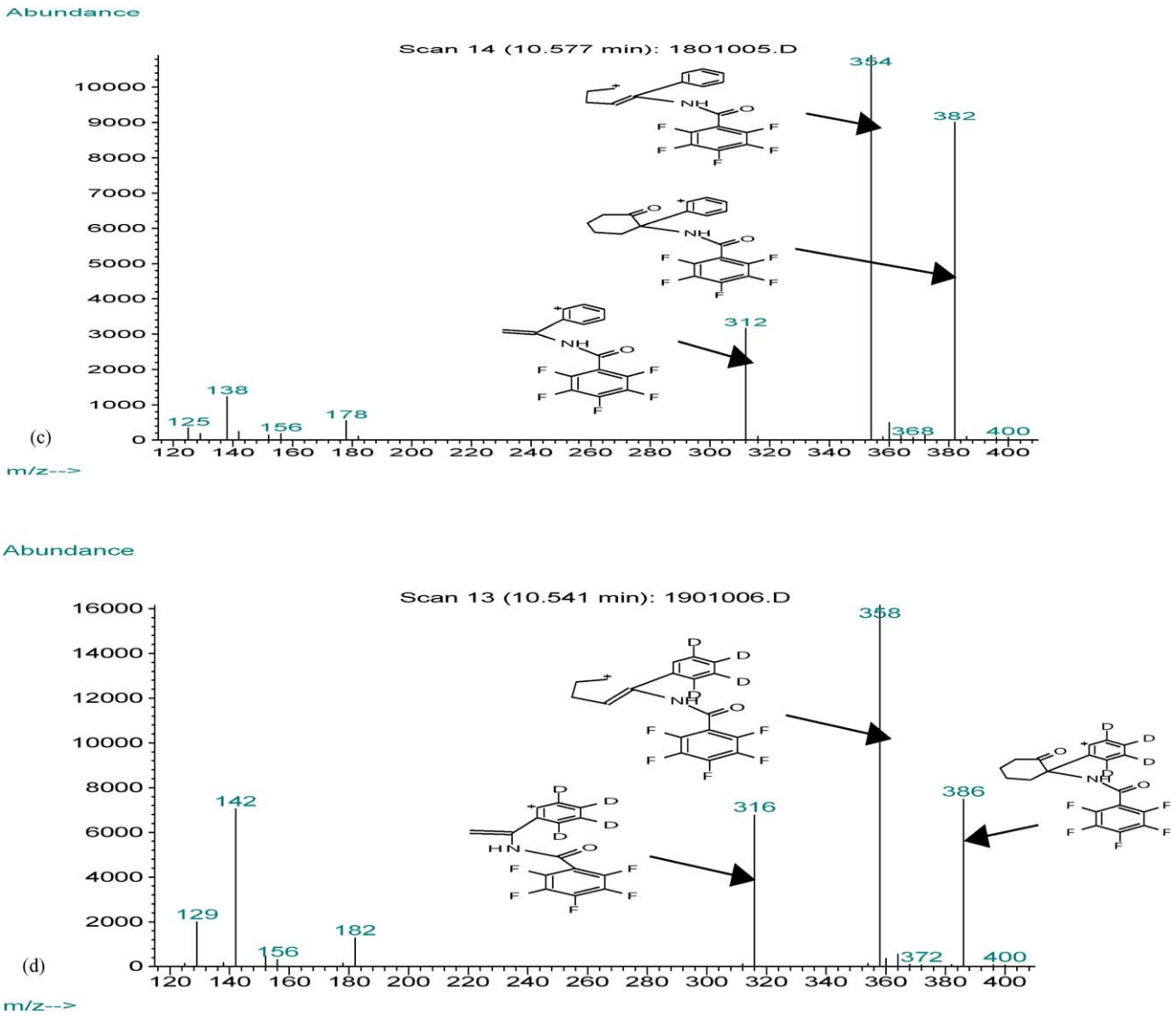

Fig. 3. (*Continued*).

spikes containing 1000, 500, 250, 100, 50, 25, 10 ng/ml, etc., of KT and NK, the respective lowest concentrations of the two analytes that analyzed accurately within ±30% of their respective target concentrations were designated as the respective LODs of the two analytes, the string being that two ion ratios of each PFBC derivative (i.e., $I_{m/z\,152}/I_{m/z\,360}$ and $I_{m/z\,368}/I_{m/z\,360}$ for PFB–KT, and $I_{m/z\,312}/I_{m/z\,354}$ and $I_{m/z\,382}/I_{m/z\,354}$ for PFB–NK, taking GC–EIMS approach as the example) matched within ±20% of those of the calibrators. In turn, the LOQs were the respective lowest concentrations of the two analytes that quantitated within ±20% of their respective target concentrations, the string being that the above stated two ion ratios of each PFBC derivative also matched within ±20% of those of the calibrators. Thus, listed on the left of Table 3 are the LODs and LOQs resulting from the serial analyses of KT and NK, ranging from 5 to 15 ng/ml for GC–EIMS approach and 50–100 ng/ml for GC–PCIMS approach. The LODs calculated for the GC–EIMS approach, i.e., 10 and 5 ng/ml, are close to the values, 20 and 10 ng/ml, previously reported for the determination of KT/NK enantiomers in plasma using chiral GC with electron capture detection [32]. In contrast,

Table 3
Method limits of detection (LODs) and method limits of quantitation (LOQs) for the total analysis of KT and NK in urine via GC–EIMS and GC–PCIMS, respectively

Ionization mode	Analyte	Definition A[a]		Definition B[a]	
		LOD (ng/ml)	LOQ (ng/ml)	LOD (ng/ml)	LOQ (ng/ml)
EI	KT	10	15	4	13
	NK	5	10	3	9
PCI	KT	75	100	50	75
	NK	50	75	30	50

[a] Definitions A and B and their criteria are given in the text.

definition B is somewhat academic [40]. Nevertheless, its relevant data are presented in support of the practicability of the title drug-testing scheme. Here the two limits are defined as the analyte concentrations giving peaks in the EIC with heights equal to the mean + $N \times$ standard deviation, where $N = 3$ for the LOD and 10 for the LOQ. The mean is the measured average of noises taken from a baseline region located far away from the analyte peak using a fortified sample. Accordingly, the standard deviation is the measured fluctuations of the noises. Our fortified sample was made by adding 50 μl of authentic 100 μg/ml KT/NK binary solution and 20 μl of 100 μg/ml KT-d_4/NK-d_4 binary IS solution to 1 ml of blank urine, and was pretreated and analyzed according to the above described procedure. Shown on the right of Table 3 are the LODs and LOQs calculated for the respective quantifier ions using an HP MS Chemstation software. The LODs calculated for the GC–EIMS approach, i.e., 4 and 3 ng/ml, are comparable with a previously reported value, 5 ng/ml, achieved using a different GC–MS-based method [21]. Overall, NK has lower LODs and LOQs than KT, once again suggesting that NK is easier to PFBC derivatize than KT. At least partially because the GCQ instrument used in this study for the GC–PCIMS analyses was not in its optimal form, the resulting LODs and LOQs were all poorer than those obtained via the GC–EIMS approach. Nevertheless, the generally low limits achieved in this study should sufficiently meet the future requirements of most of the urine drug testing programs and even the criminal cases in Taiwan, ROC, where amphetamines must not be detected, i.e., zero tolerance.

3.5. Analyte recoveries indicative of method performance

In order to achieve an immunity to a wide variety of chemical and physical interferences and to improve the quantitative analytical quality, isotope-dilution method was

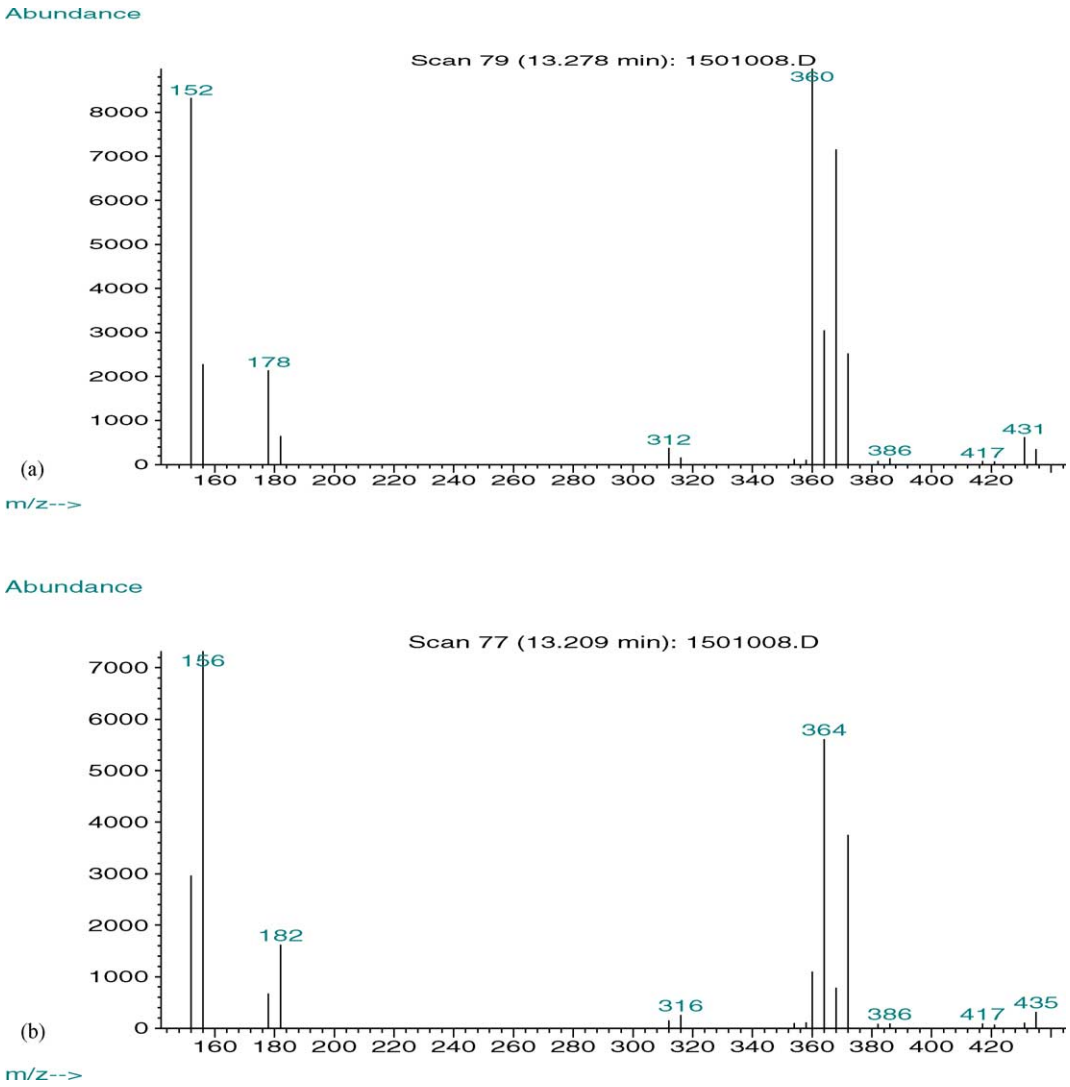

Fig. 4. The cross-contaminated mass spectra of (a) PFB–KT and (b) PFB–KT-d_4 (fetched from the right-hand peak in Fig. 1), and those of (c) PFB–NK and (d) PFB–NK-d_4 (fetched from the left-hand peak in Fig. 1). Only the key mass region is shown.

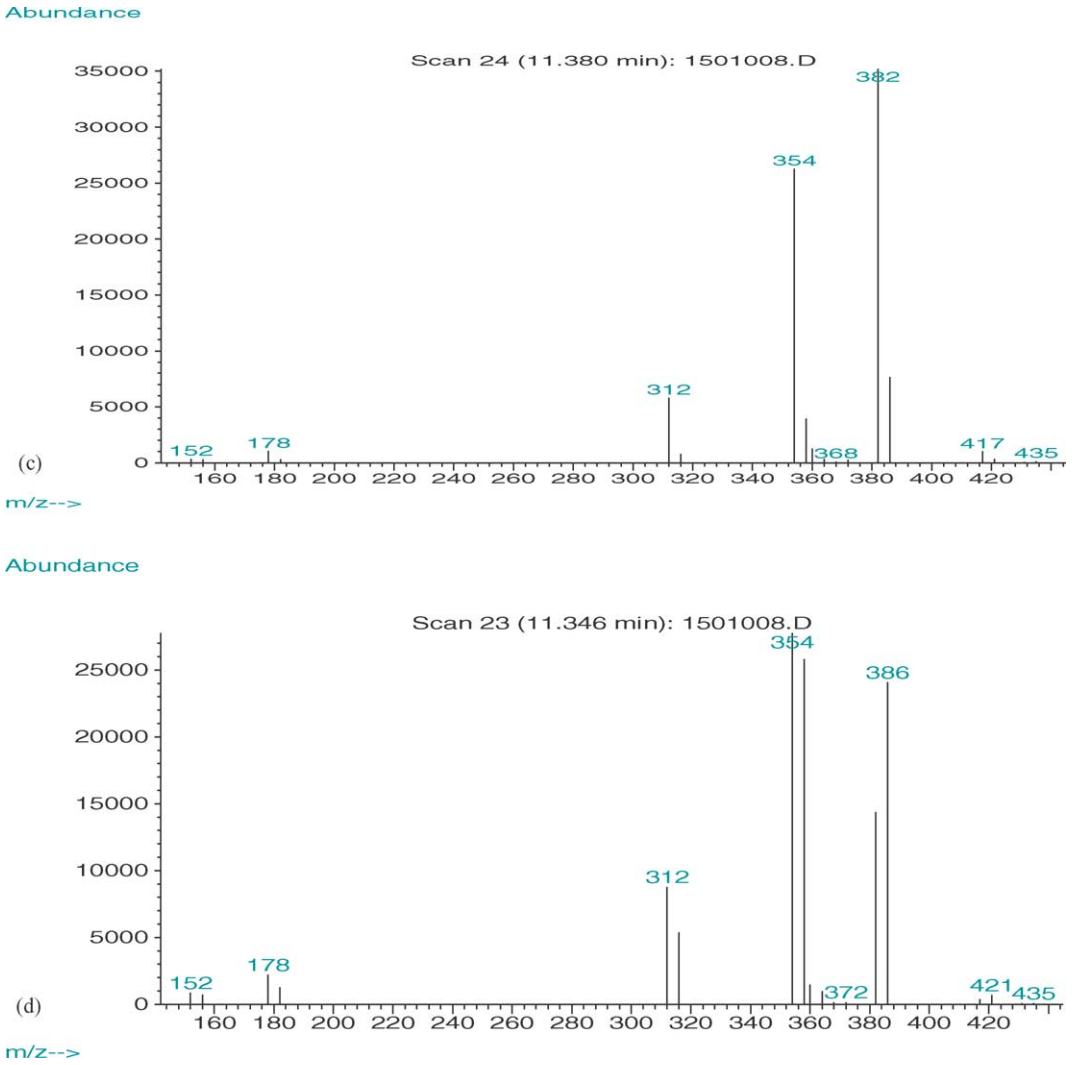

Fig. 4. (*Continued*).

employed in this study. As is described in Section 2.2, known amounts of d_4-labeled ISs were routinely added to the urine sample prior to performing LLE. Nevertheless, in addition to the full understanding of the instrumental performance, it is also informative to have insight into the actual efficiency of LLE and ChD.

A pair of instrumental calibration curves were plotted in advance with the equations being $y = 0.0087x + 0.0012$ for KT and $y = 0.0119x - 0.1464$ for NK, using appropriate authentic KT·HCl/KT·HCl-d_4/NK·HCl/NK·HCl-d_4 solution in EA. Two aliquots (50 and 100 μl) of a 100 μg/ml KT/NK urinary spike were subjected, respectively, to LLE followed by PFBC–ChD and GC–EIMS SIM analysis according to the procedure described in Section 2.2.2. The recoveries of KT and NK were obtained by dividing the regressed concentrations of recovered KT and NK (actually recovered in the form of PFBC derivatives) by the originally spiked concentrations of KT and NK, i.e., 100 μg/ml. Thus, the mean recoveries calculated for the 50 μl aliquot were 71.0 ± 2.5% for KT and 96.5 ± 2.1% for NK, and that for the 100 μl aliquot, 71.6 ± 2.3% for KT and 97.8 ± 2.6% for NK, all based upon triplicate analyses. The fair to high recoveries and good precisions indicate that the whole analytical scheme including the LLE, ChD, and GC–EIMS SIM is effective, reliable, and robust. As had been expected, NK gave higher recoveries than KT.

3.6. Auxiliary GC–MS analysis using PCI

If necessary, GC–MS analysis using CI can provide both qualitative and quantitative information complementary to those given by the prevalent EI mode. For the detection of PFB–KTs, PCI was found to produce instrumental responses nearly 10 times those given by negative ion CI (NCI), being in agreement with our previous experience in analyzing amphetamines. As shown above in Fig. 6 for the GC–PCIMS mass spectra of the four PFB–KTs, the respective quasi-molecular ion peaks ($m/z = M + 1$, $M + 3$) are always prominent and characteristic. The problem of mass-spectrum resemblance between the analyte and its IS

due to the mutual mix-up of fragmented ions caused by the inadequate resolution of full-scan TIC peaks was not so serious as that with the EI counterparts. By using the highly characteristic and little cross-contributed quasi-molecular ions as qualifier and/or quantifier ions, the effective resolution and hence the accuracy and precision for the GC–PCIMS analysis can still be secured. Furthermore, unlike the foregoing EI mass spectra resulting from relatively "hard" ionization, the mass spectra obtained by the "softer" PCI ionization can always distinctly reflect the natural-abundance ratio of ^{35}Cl and ^{37}Cl isotopes, i.e., $I_{m/z\,M+1}/I_{m/z\,M+3} \fallingdotseq 3{:}1$, thereby facilitating the confirmation of KTs. In Fig. 6, the $m/z\ M+1$ ions for the four PFB–KTs are 431.6, 435.6, 417.5, and 421.5, respectively, and the $m/z\ M+3$ ions are 433.6, 437.6,

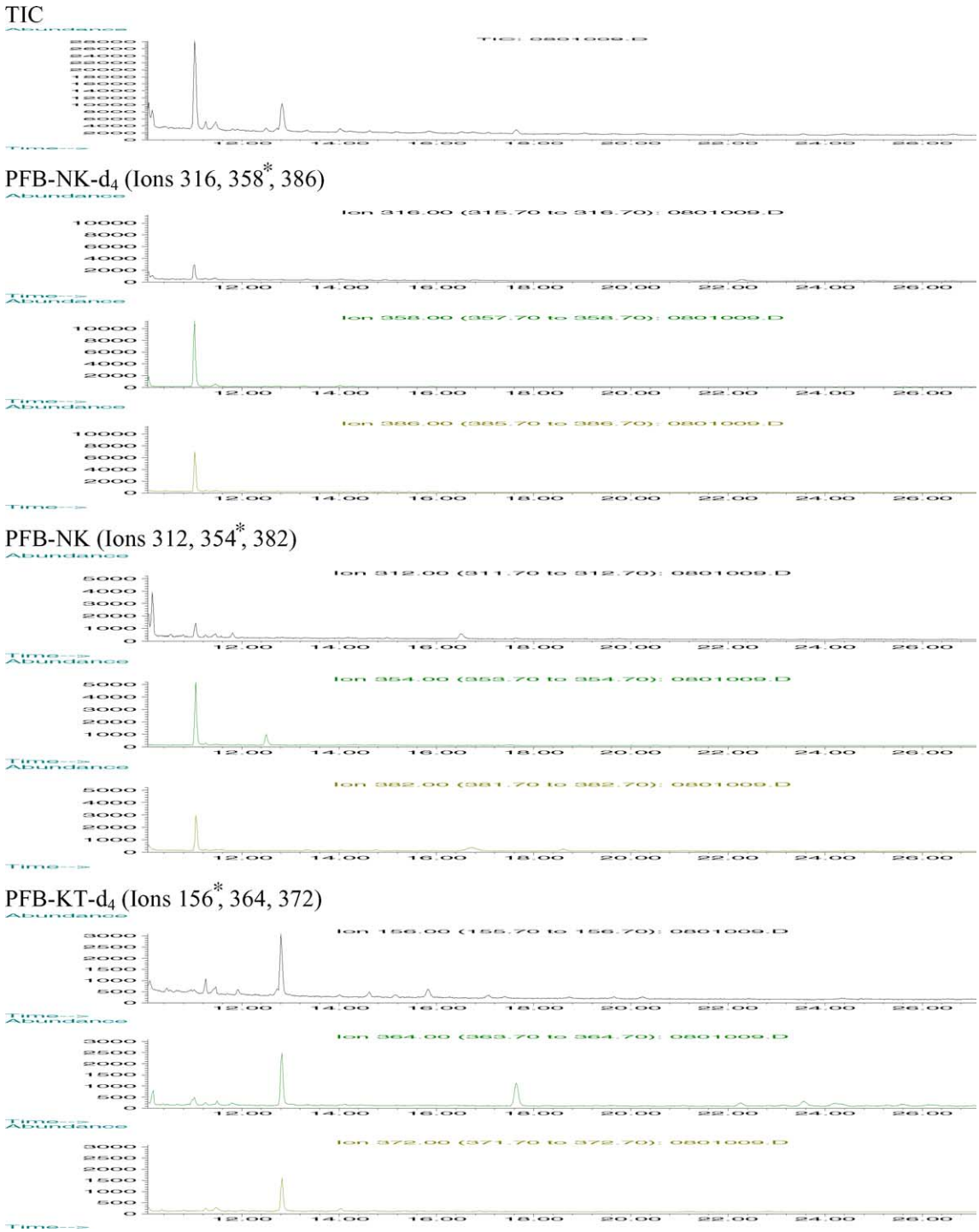

Fig. 5. Reconstructed GC–EIMS SIM chromatograms resulting from the total analysis (first run) of real-case sample 1 in Table 4 which quantified 1134 ng/ml of KT and 847 ng/ml of NK. The asterisked ions are the respective quantifier ions.

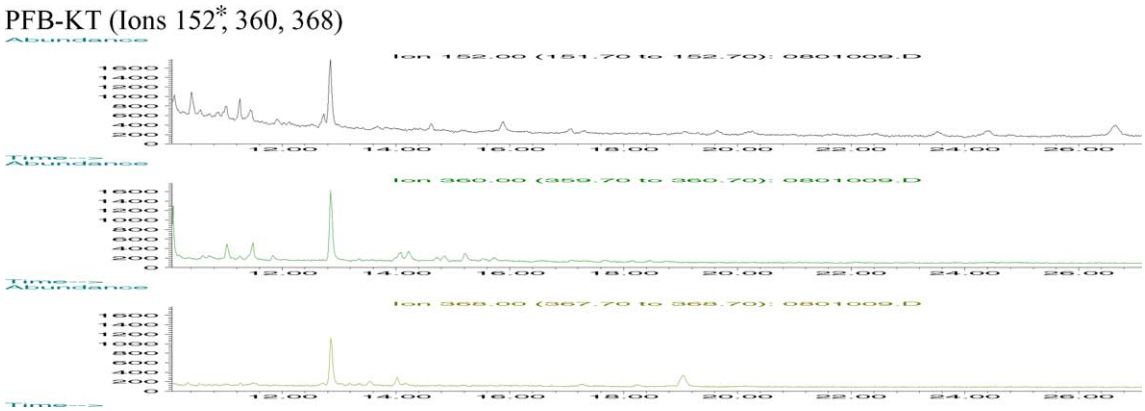

Fig. 5. (*Continued*).

419.6, and 423.6, respectively. The small decimal deviations from the respective nearest whole numbers should have been covered by a 0.5-window in the whole-numbered mass monitoring.

3.7. Case study

The analytical scheme proposed in this report as a choice of confirmatory protocol for forensic urine drug testing was

Fig. 6. The mass spectra of (a) PFB–KT, (b) PFB–KT-d_4, (c) PFB–NK and (d) PFB–NK-d_4 obtained upon the GC–PCIMS full scan of a pretreated urinary spike containing all the four PFB–KTs. Only the key mass region is shown.

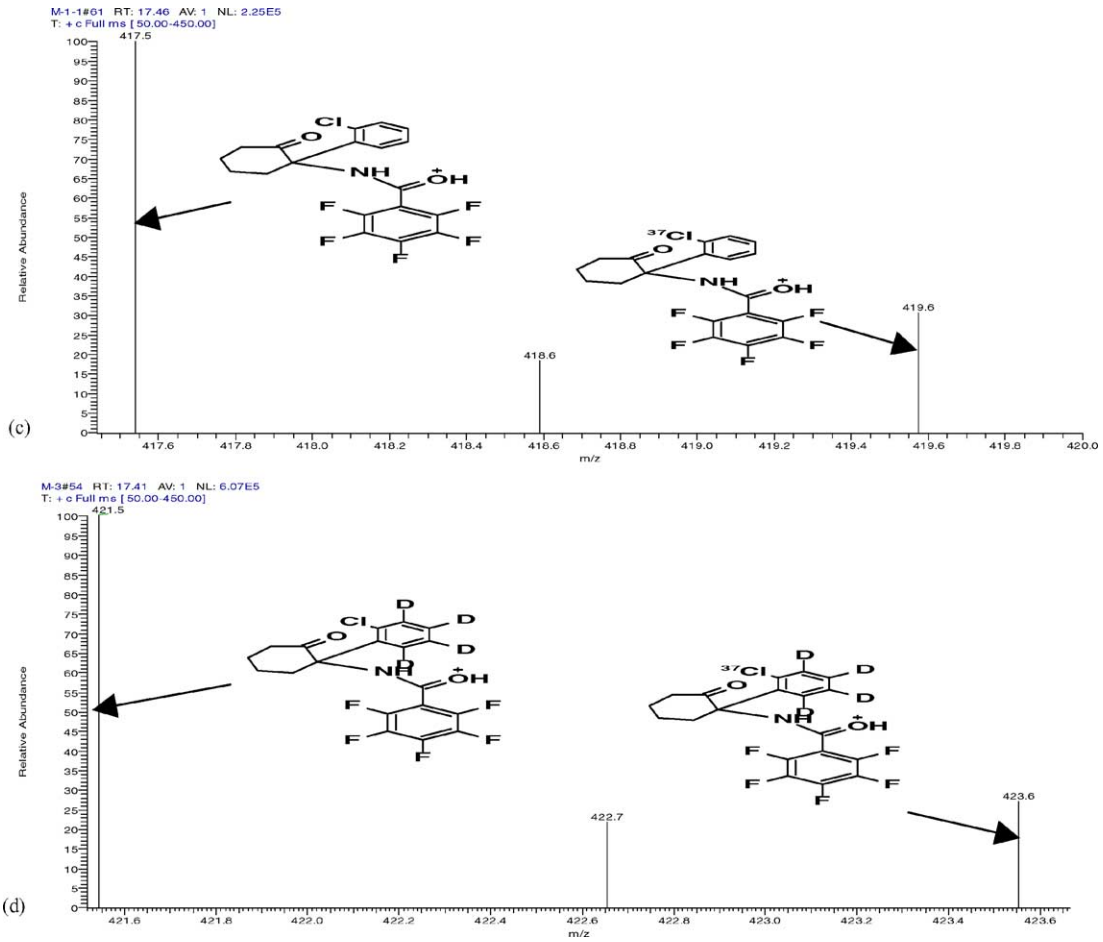

Fig. 6. (*Continued*).

Table 4
Results obtained upon the total analyses of four real-case samples, all definitely involving KT administration

No. of sample	No. of run	KT (ng/ml)		NK (ng/ml)	
		GC–EIMS	GC–PCIMS	GC–EIMS	GC–PCIMS
1	1	1134	1080	847	776
	2	1158	905	913	825
	3	1225	972	889	694
		$\bar{X} = 1172$	$\bar{X} = 986$	$\bar{X} = 883$	$\bar{X} = 765$
		CV = 4.0%	CV = 9.0%	CV = 3.8%	CV = 8.7%
2	1	345	308	1711	1677
	2	355	321	1689	1545
	3	336	289	1723	1309
		$\bar{X} = 345$	$\bar{X} = 306$	$\bar{X} = 1708$	$\bar{X} = 1510$
		CV = 2.8%	CV = 5.2%	CV = 1.0%	CV = 12.3%
3	1	223	183	54[a]	43[a]
	2	214	178	54[a]	40[a]
	3	231	199	52[a]	49[a]
		$\bar{X} = 223$	$\bar{X} = 187$	$\bar{X} = 53$	$\bar{X} = 44$
		CV = 3.8%	CV = 5.9%	CV = 1.8%	CV = 10.0%
4	1	185	157	342	324
	2	167	138	330	287
	3	174	142	340	315
		$\bar{X} = 175$	$\bar{X} = 146$	$\bar{X} = 337$	$\bar{X} = 309$
		CV = 5.2%	CV = 6.4%	CV = 2.0%	CV = 5.7%

[a] Obtained by extrapolation.

applied to the determination of KT and NK in four real urinary specimens that "might" have involved the administration of KT. No more information about the screening method or data was given. After getting through the above described analytical procedure and regression calibration in triplicate, the relevant data were displayed in Table 4. Although samples 2–4 presented in their GC–EIMS SIM chromatograms (unshown) a few more irrelevant peaks than does the neatest sample 1 (Fig. 5), those extra peaks turned out to be non-interfering with the highly specific monitoring of the analytes/ISs. Putting aside the forensic interpretation of the drug levels, several low outcomes and all the small CV values (typically below 12.3%) have validated the proposed analytical scheme as a competent confirmatory protocol for forensic urine KT testing. Once again, at least partially because the GCQ instrument used in this study for the GC–PCIMS analyses was not in its optimal form, the relevant results were all poorer than those obtained via the GC–EIMS approach.

4. Conclusions

The results presented in this report demonstrated that LLE and ChD followed by isotope dilution GC–MS is a sound analytical scheme for the conclusive determination of KT and NK in urine, and should meet the possible criteria to be adopted by the US HHS and Department of Defense (DoD) drug testing programs in the near future. The simultaneous derivatization of the primary-amine NK and secondary-amine KT with PFBC has not only enhanced their instrumental responses and mass-spectrum uniqueness but also afforded more proper yet easier selection of qualifier and quantifier ions and hence achieved more evidential identification and quantitation. GC–MS operated in the PCI mode can offer both qualitative and quantitative information complementary to those given by the EI mode. However, in this study the results produced via the GC–PCIMS approach are generally not so good as those achieved via the GC–EIMS approach, suggesting that the latter approach be the primary choice and the former a supplement. The proposed scheme is simple, effective, reliable, and robust. It may serve as a confirmatory protocol for forensic urine drug testing. Interestingly, during the course of this study, a report on the confirmatory analysis of KT and NK in urine using LC-isotope dilution MS appeared in the literature [15]. However, the authors did not prepare derivatized samples, presumably because LC is based on different instrumental theories and operated in milder operational conditions.

Due to its commercially unavailability, we did not incorporate DHNK into the analyte list. As a matter of fact, it has been reported based on a parallel LC–MS study that the high temperatures of GC or GC–MS may lead to falsely higher DHNK concentrations [15]. It has also been discussed by way of preparing rat liver microsomes that DHNK is biochemically formed in only small amounts and any larger amounts found could have been an artifact of the work-up procedures used [16]. We shall undertake a close follow-up in these matters.

Acknowledgements

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