ORIGINAL ARTICLE

# Forensic Toxicological Analysis of Ketamine and Norketamine in Fingers and Toenails using LC-QTOF-MS

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## Abstract

**Background:** Human nails are primarily composed of keratin, a fibrous protein. As nails grow, drugs that have been misused can become embedded in the keratin fibers, allowing detection even months after use. This prolonged retention has led to increased interest in utilizing keratinized matrices for both forensic and therapeutic purposes. Unlike traditional biological samples such as blood and urine, which have shorter detection windows, keratinized matrices can preserve evidence of repeated drug exposure for extended periods.

**Materials and Methods:** This study analyzes nail clippings from five individuals with documented ketamine (KET) misuse. Before liquid chromatography-mass spectrometric analysis, the samples underwent a series of preparatory steps, including decontamination, hydrolysis in 1 M sodium hydroxide at 90°C for one hour with sonication, extraction using ethyl acetate, and reconstitution in methanol.

**Results:** Calibration curves were created using reference standards, covering KET concentrations from 0.20 ng/mg to 16 ng/mg and norketamine (NKT) concentrations from 0.30 ng/mg to 16 ng/mg. The limits of quantification and detection for KET in spiked nail clippings were found to be 0.2 ng/mg and 0.05 ng/mg, respectively. For NKT, the respective values were 0.07 ng/mg and 0.15 ng/mg.

**Conclusion:** This study presents a targeted analytical method for detecting KET and its metabolite, NKT, in human nails. The proposed approach could be valuable for applications such as monitoring drug misuse, investigating drug-facilitated crimes, and assessing cases of medical negligence.

**Key words:** Drug abuser, fingernail, forensic toxicology, ketamine and norketamine, toenail

## INTRODUCTION

Forensic toxicology is an interdisciplinary field dedicated to detecting and analyzing drugs and other harmful substances in bodily fluids and tissues. The primary purpose of forensic drug testing is to identify drug use in individuals and to deter or prevent future substance abuse.[1] Drug abuse is currently a major global health and socioeconomic concern. Forensic toxicologists are often tasked with analyzing biological samples from individuals suspected of drug use and interpreting the results in relation to complex behaviors, such as drug-facilitated sexual assault and driving under the influence.[2]

In forensic chemistry and toxicology, blood, urine, oral fluid, and sweat have traditionally been the most used matrices for drug testing. However, alternative matrices offer several advantages over conventional blood and urine analysis.[3,4] This has led to the increasing popularity of drug monitoring in keratinized matrices, such as human nails and both scalp and non-scalp hair. These matrices offer advantages, including an extended detection window ranging from weeks to years, enabling the identification of xenobiotic exposure or substance ingestion.[2,3,5] In addition, these advantages include being

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non-invasive, easy to use, not requiring medical expertise, and allowing for collection under direct observation to prevent adulteration. Human nails can also be stored and transported at room temperature. Additionally, studies have shown that drug concentrations are generally higher in fingernails compared to toenails, making fingernails a more sensitive matrix for detecting drug exposure in forensic toxicology[7] with studies comparing drug concentrations between hair and nails.[8] These benefits also apply to hair, another keratinized matrix that has been used for several years to assess past drug consumption.[8] Interestingly, nails offer several additional advantages over hair. One key benefit is that nails lack melanin, unlike hair, where drug incorporation can be influenced by melanin concentration. This makes nails particularly useful in cases where hair is unavailable, such as in individuals with alopecia, those undergoing chemotherapy, or newborns in their 1st few weeks or months of life.[9,10] In addition, since nails grow more slowly than hair, they allow for the detection of drug exposure over longer periods or at lower concentrations. Furthermore, nails grow at a consistent rate, making data interpretation more straightforward compared to hair, which follows a cyclic growth pattern with varying phases.[3,6] Finally, nail collection is easier, less invasive, and more socially acceptable than hair sampling. Collectively, these advantages emphasize the potential of nails as a valuable and effective matrix for the retrospective detection of drug use.[3,6] Consequently, this study focused on analyzing ketamine (KET) and its metabolite, norketamine (NKT).

### KET

KET, chemically known as 2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone [Figure 1], is commonly referred to by various street names, including K, K-Hole, Kate, and Special K. In the liver, KET undergoes extensive metabolism through N-demethylation, producing NKT as its primary metabolite [Figure 2]. Due to its dissociative and hallucinogenic properties, KET is primarily used in medical settings as an anesthetic or analgesic. However, its misuse has been on the rise in recent years, contributing to drug-facilitated crimes, such as sexual assaults, robberies, kidnappings, smuggling, and other illegal activities.[11,12] The World Drug Report 2023 highlights a persistent market for the non-medical use of KET in East and Southeast Asia, where its misuse continues to increase.[2]

The detection of KET and its metabolite, NKT, in nails, is primarily performed using gas chromatography, liquid chromatography (LC), and mass spectrometry (MS) or tandem mass spectrometry (MS-MS), which are widely recognized analytical methods in forensic and toxicological research.[13-18] Previous studies have analyzed KET and NKT alongside other substances, such as MDMA, MDA, and methamphetamine, using various analytical techniques. The objective of this study was to develop a simple Liquid Chromatography Quadrupole-Time of Flight-Mass

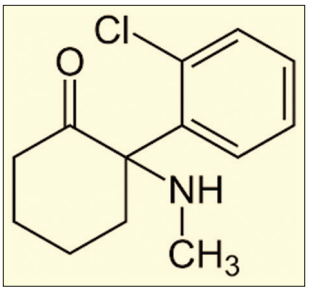


**Figure 1:** Structure of ketamine

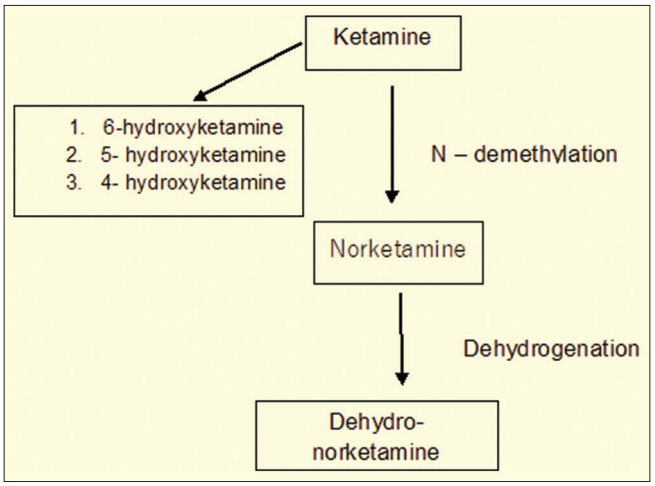


**Figure 2:** Metabolic pathway of ketamine

Spectrometry (LC-QTOF-MS) method for detecting and quantifying KET and NKT in nails at the ng/mg level. A targeted method specifically for these compounds was established and successfully applied to real nail samples collected from drug users. The method was validated using a comprehensive set of validation parameters, enabling the qualitative and quantitative analysis of KET and its metabolite, NKT.

## MATERIALS AND METHODS

### Specimens

Finger and toenail (FN and TN) clippings were collected from five participants (substance abusers) who were admitted for therapeutic intervention at a rehabilitation facility known as Parivartan Nasha Mukti Kendra, located in Ujjain, Madhya Pradesh, India. The samples of nail clippings were acquired by excising the surplus overhang of the nail plate from all ten digits to mitigate ambiguity in the concentration levels of KET and NKT in the nails, which may be influenced by varying rates of nail growth, the consumption of multiple substances, and other contributing factors. The drug abusers (participants) exhibited a documented history of KET misuse. Control nail samples were gathered from healthy volunteers for the purpose of method development.

### Reagents and materials

NKT and KET reference standards were purchased from Sigma Aldrich and High-performance liquid chromatography-grade ethyl acetate, acetonitrile, and methanol from S.K. Trader in Indore, India. These chemicals were stored in a deep freezer until needed.

### Sample preparation protocol

Initially, the mass of each FN and TN clipping was measured. The samples then underwent a thorough cleaning process, which involved washing with 5 mL of water for 15 min, repeated three times. Subsequently, a methanol cleaning procedure was performed using an ultrasonicator to remove any potential contaminants. After being left to dry overnight at ambient temperature, 50 mg of the samples were precisely weighed and transferred into a sterile test tube. To extract the analytes, the samples were incubated at 90°C for one hour in the presence of 1 mL of 1.0 M sodium hydroxide solution. The extraction process was carried out using 5 mL of ethyl acetate, followed by centrifugation at 6000 rpm for 5 min. The organic supernatant was then carefully transferred to a new test tube. After drying, the organic phase was reconstituted with 1 mL of methanol. A validated analytical technique was applied to quantify various analytes using an Agilent Q-TOF G6550B LC coupled with an Agilent 1260 Infinity II LC.

**Table 1:** Conditions for the chromatographic gradient

| Time (min) | % Mobile Phase A | % Mobile Phase B |
|---|---|---|
| 2 | 90 | 10 |
| 8 | 10 | 90 |
| 12 | 10 | 90 |
| 12.50 | 90 | 10 |
| 15 | 90 | 10 |

### LC-MS procedure

A Poroshell 120 EC-C8 LC column with dimensions (100 mm × 3.0 mm × 2.7 µm) was used throughout the analysis. The optimized mobile phase consisted of 0.1% formic acid in acetonitrile, applied under gradient mobile phase conditions [Table 1] at a flow rate of 0.4 mL/min, with a total run time of 15 min. Detection was performed using Q-TOF-LC/MS (Agilent G6540B) instruments in both full scan mode and targeted MS/MS mode, utilizing electrospray ionization in the positive ionization state. The mass spectral parameters were optimized to achieve the best results, with an ion spray voltage of 3500 V, a heater temperature of 290°C, nebulizer gas ($N_2$) at 30 psi, and collision-activated dissociation gas in the collision cell at 8 psi. The molecular ions (parent ions) of KET and NKT were determined to be 238 and 224, respectively, corresponding to the $[M+H]^+$ ions. Two major product ions were monitored simultaneously: 125 and 179 for KET, and 179 and 217 for NKT. Among these, ion 125 for KET and ion 217 for NKT were selected as the quantifier ions.

## RESULTS

### Validation of analytical method

The separation process has been optimized in full scan mode of the LC-QTOF-MS. The selected column offers remarkable reliability as well as effectiveness. After multiple attempts, gradient elution from 90% to 10% B for 15 min was used, with eluents A and B being 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. Mass spectra for the pre-cursor ion (molecular ion) were obtained using the mass analyzer's pre-cursor ion scan mode at quadrupole 1 (Q1). The molecular ion for KET [Figure 3] is 338[M+H], while for NKT [Figure 4], it is 224[M+H]. Similarly, the product ion spectrum and fragmentation of the molecular ions 338 and 224 are carried out. Figures 5 and 6 illustrate the product

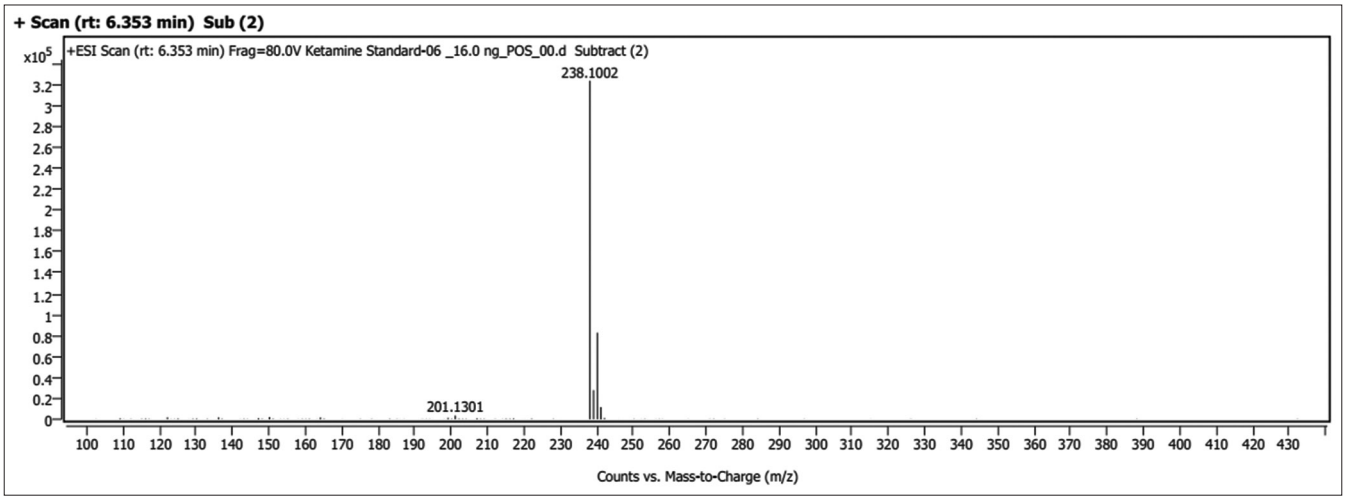


**Figure 3:** The mass sepectrogram of the ketamine

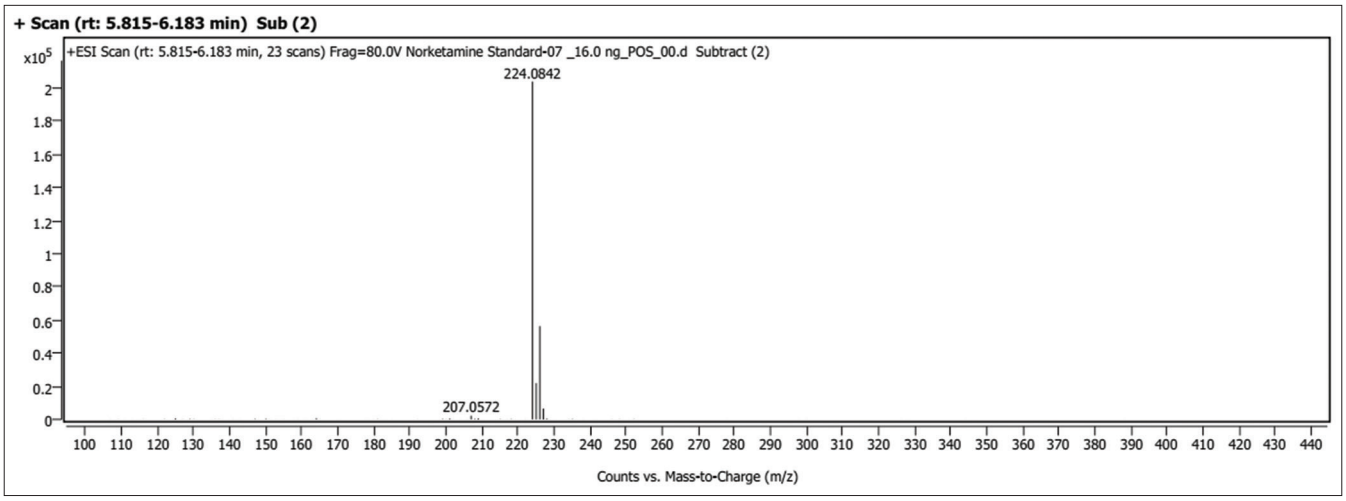


**Figure 4:** The mass chromatogram of norketamine

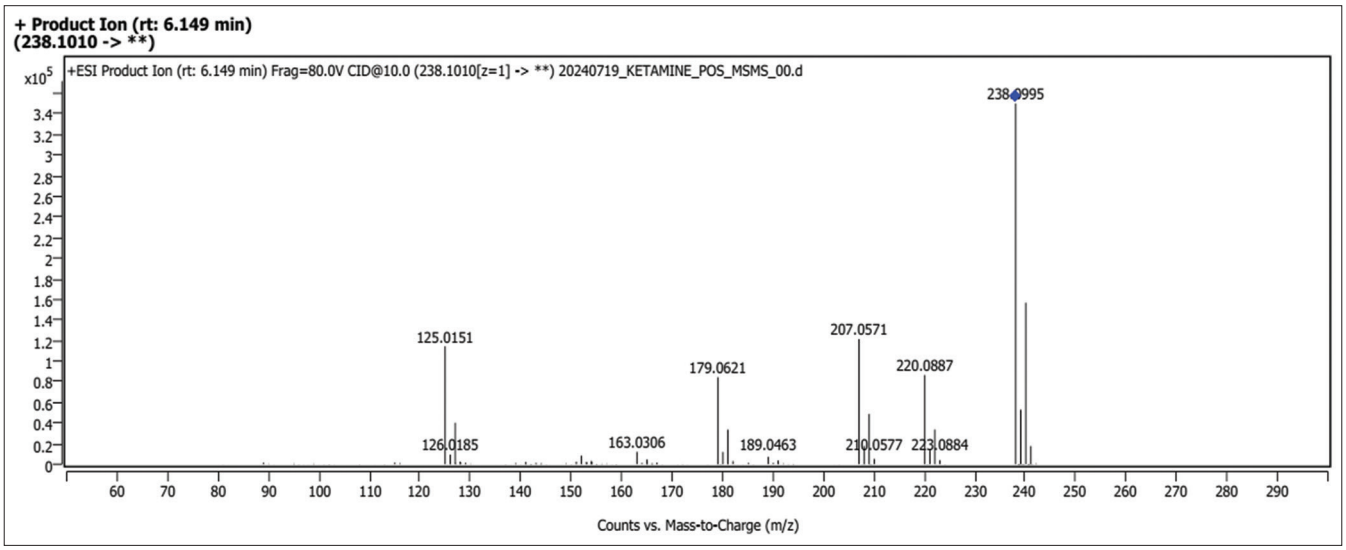


**Figure 5:** Ketamine's product ion mass spectrum

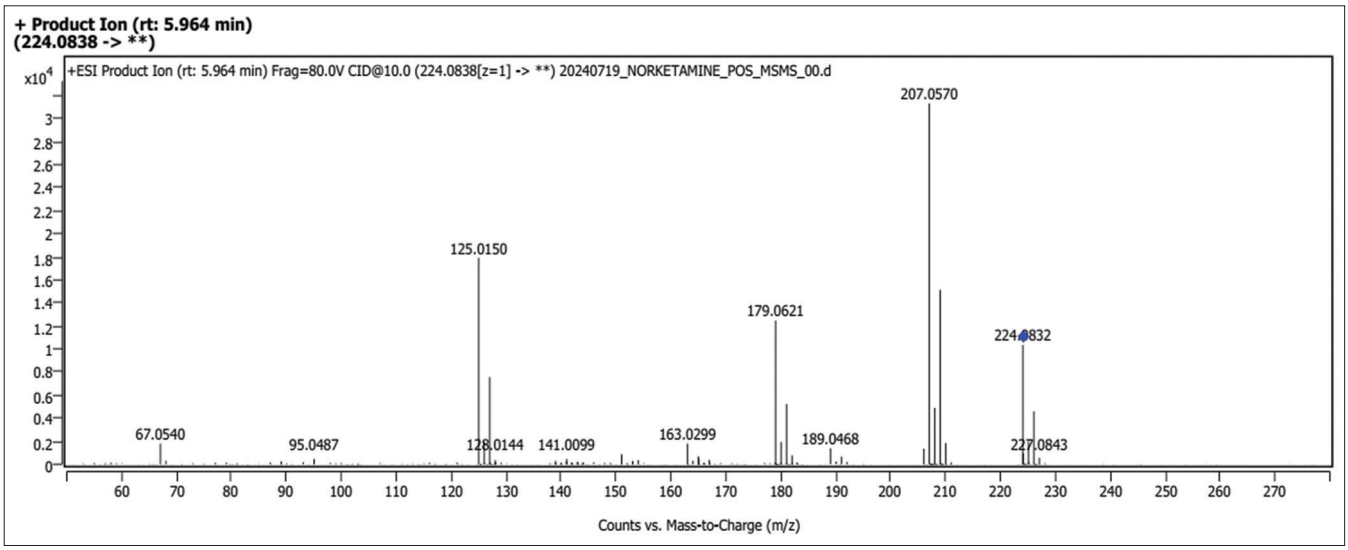


**Figure 6:** Norketamine's product ion mass spectrum

ion spectrum of the ions 338 (KET) and 224 (NKT), with 220 being the most abundant ion (base peak) and 207 being the next most abundant ion for KET [Figure 5]. Similarly, the most abundant ion (base peak) for NKT was 207, which was followed by 125 [Figure 6].

The method was validated in terms of linearity, precision, accuracy, specificity, selectivity, detection and quantification limits, and sensitivity.

## Studies on method validation

### *Selectivity*

Selectivity was determined by analyzing blank samples and determining the absence or presence of peaks during the drug's retention time.

### *Linearity*

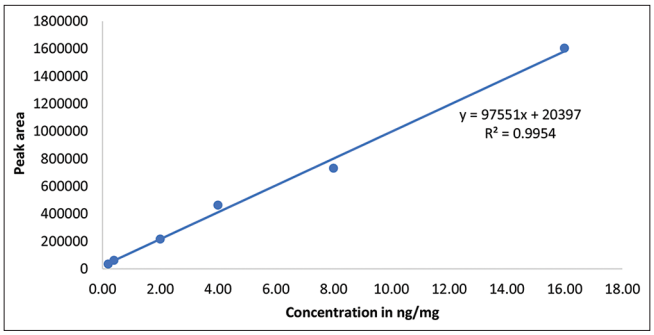


**Figure 7:** Linearity calibration curve of ketamine spiked solution in nail clipping

Following the extraction and LC-MS analysis of 70 mg of real spiked blank nail clipping, which contained 0.20 ng/mg to 16 ng/mg of KET and 0.30 ng/mg to 16 ng/mg of NKT reference standard, a standard calibration curve was plotted [Figures 7 and 8; Table 2].

### *Precision and accuracy*

The relative standard deviation of repeated measurements was employed to evaluate the precision of the method. The standard deviation of both nominal and measured concentrations was utilized for the assessment of accuracy. This was accomplished by examining samples that were spiked at two distinct concentrations: 4 and 16 ng/mg of NKT, as well as for KET. To assess intraday repeatability, three replicates were implemented. Triplicate analyses were performed at these concentrations over a 3-day duration to calculate the inter-accuracy [Tables 3-6].

## Quantification of KET and NKT from fingers and TN samples

The quantities of KET and NKT detected in the samples of FNs and TNs were derived from the calibration curve established using standard solutions of KET and NKT. The respective concentrations are presented in [Tables 7 and 8] for both the analyzed FN and TN samples.

The chromatograms and mass spectra acquired during the LC-QTOF-MS analysis for one FN (S3FN-13) and one TN (S3TN-13) sample in positive ion mode are illustrated in [Figures 9-12] for KET and in [Figures 13-16] for NKT, respectively.

**Table 2:** Data on sensitivity and linearity of the analytes under study in nails

| Analyte | Linear regression | Determination coefficient ($r^2$) | LOD | LOQ |
|---|---|---|---|---|
| Ketamine | y=97551x+20397 | 0.9954 | 0.05 ng/mg | 0.2 ng/mg |
| Norketamine | y=295185x+73932 | 0.9969 | 0.07 ng/mg | 0.15 ng/mg |

LOD: Limit of detection, LOQ: Limit of quantification

**Table 3:** Data on the developed method's accuracy and precision (intraday) for norketamine

| Matrix | Added (ng/mg) | Intraday (*n*=3) | | |
|---|---|---|---|---|
| | | Found concentration±SD (ng/mg) | RSD | Recovery (%) |
| Nail | 4 | 3.92±0.25 | 6.38 | 98 |
| | 16 | 15.78±0.27 | 1.72 | 98.62 |

**Table 4:** Data on precision and accuracy of the developed method for norketamine (interday studies)

| Matrix | Added (ng/mg) | Interday (n=3) | | |
|---|---|---|---|---|
| | | Found concentration±SD (ng/mg) | RSD | Recovery (%) |
| Nail | 4 | 3.85±0.26 | 6.88 | 89.5 |
| | 16 | 15.84±0.52 | 3.21 | 99 |

SD: Standard deviation, RSD: Relative standard deviation

**Table 5:** Data on the accuracy and precision (intraday) of the developed method for ketamine

| Matrix | Spiked (ng/mg) | Intraday (*n*=3) | | |
|---|---|---|---|---|
| | | Found concentration±SD (ng/mg) | RSD | Recovery (%) |
| Nail Clippings | 4 | 3.47±0.33 | 9.51 | 86.75 |
| | 16 | 15.93±0.30 | 1.88 | 99.56 |

SD: Standard deviation, RSD: Relative standard deviation

**Table 6:** Accuracy and precision data of the developed method for ketamine (Inter day)

| Matrix | Spiked (ng/mg) | Interday (*n*=3) | | |
|---|---|---|---|---|
| | | Found concentration±SD (ng/mg) | RSD | Recovery (%) |
| Nail Clippings | 4 | 3.28±0.26 | 7.92 | 82 |
| | 16 | 15.64±0.64 | 4.09 | 97.75 |

**Table 7:** Concentration of KET FN and TN samples

| Sample code | Target analyte | Concentration in FN ng/mg | Concentration in TN ng/mg |
|---|---|---|---|
| S1 | Ketamine | 1.37 | 1.07 |
| S2 | | 1.86 | 1.36 |
| S3 | | 1.58 | 1.22 |
| S4 | | 1.09 | n.d. |
| S5 | | 0.91 | 1.75 |

FN: Fingernail, TN: Toenail, KET: Ketamine, n.d.: Not detected

**Table 8:** Concentration of NKT FN and TN samples

| Sample code | Target analyte | Concentration in FN ng/mg | Concentration in TN ng/mg |
|---|---|---|---|
| S1 | NKT | n.d. | 0.53 |
| S2 | | n.d. | 0.30 |
| S3 | | 1.12 | 0.79 |
| S4 | | 0.30 | n.d. |
| S5 | | 0.21 | n.d. |

FN: Fingernail, TN: Toenail, NKT: Norketamine, n.d.: Not detected

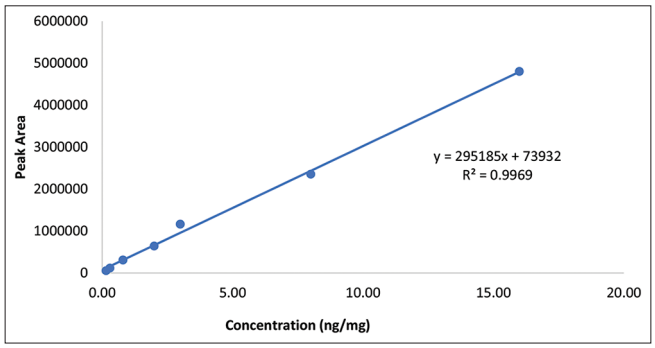


**Figure 8:** Norketamine-spiked nail clipping solution's linearity calibration curve

This paper reports the results of an LC-QTOF-MS analysis performed on five FN and TN samples obtained from drug abusers undergoing treatment at a drug rehabilitation and de-addiction center. For the purpose of developing the method, control nail samples were taken from the student volunteer. The results are presented in Tables 7 and 8. In total five samples (S1-S5) were analyzed for KET and NKT. KET was found present in FNs of all drug abusers (S1FN-S5FN) under this study. However, in TNs, KET could be detected only in four drug abusers (S1TN, S2TN, S3TN, and S5TN) TN samples. Further, it was noticed that NKT (a metabolite of KET) was identified only in three FNs (S3FN, S4FN, and 5FN) and three TNs (S1TN S2TN, and S3TN) of drug abuser's samples. It may be due to the degree of accumulation of drug is higher in FNs compared to TNs. In this investigation, it was found that the concentrations of KET in FNs and TNs were higher than those of NKT. The minimum-maximum concentration for KET and NKT in both FN and TN is mentioned in Table 9. Previous studies also reported higher concentrations of the KET as compared to NKT in nail clippings.[13,15,18] However, a study by Kim *et al.*[16] KET content in FN clippings was recorded as 0.314 ng/mg (below the limit of quantification).

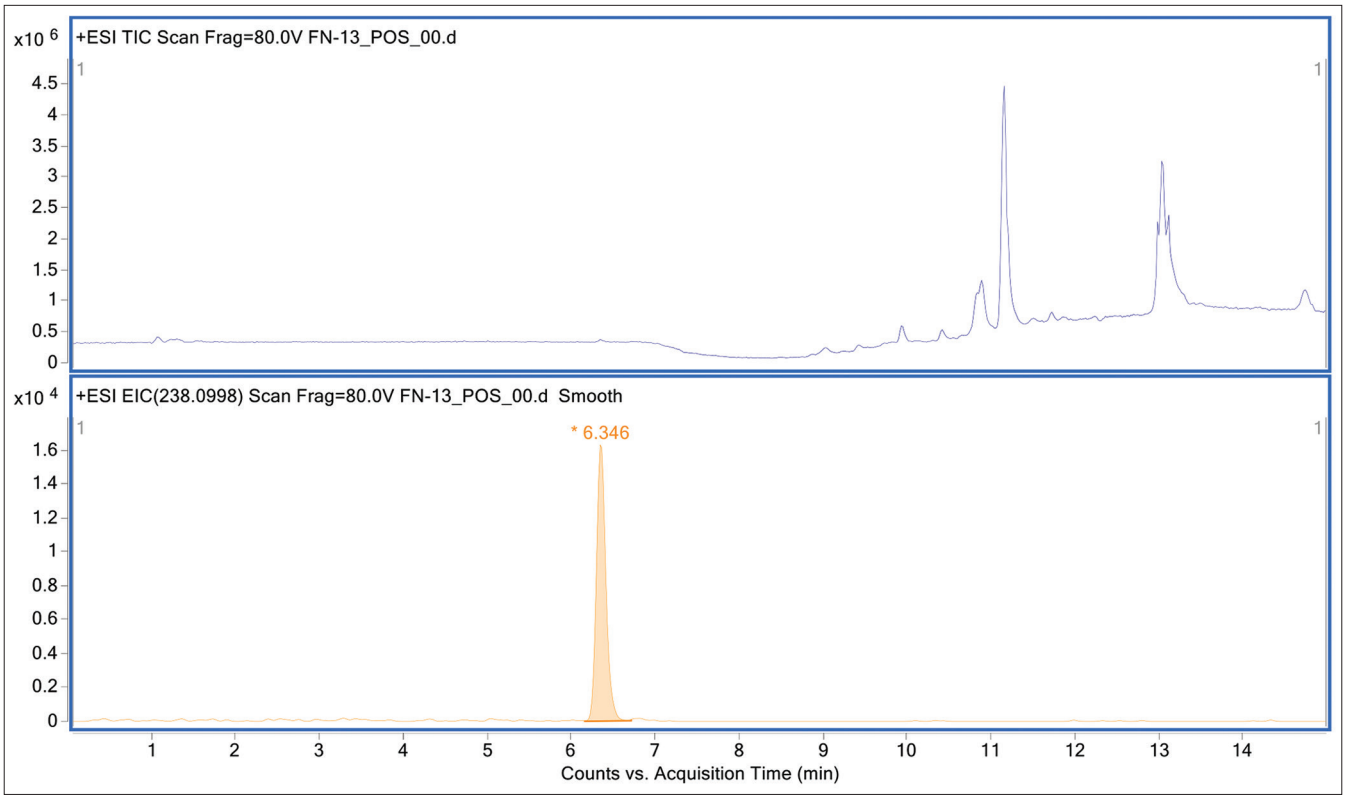


**Figure 9:** The chromatogram of S3 (Finger nail-13) ketamine

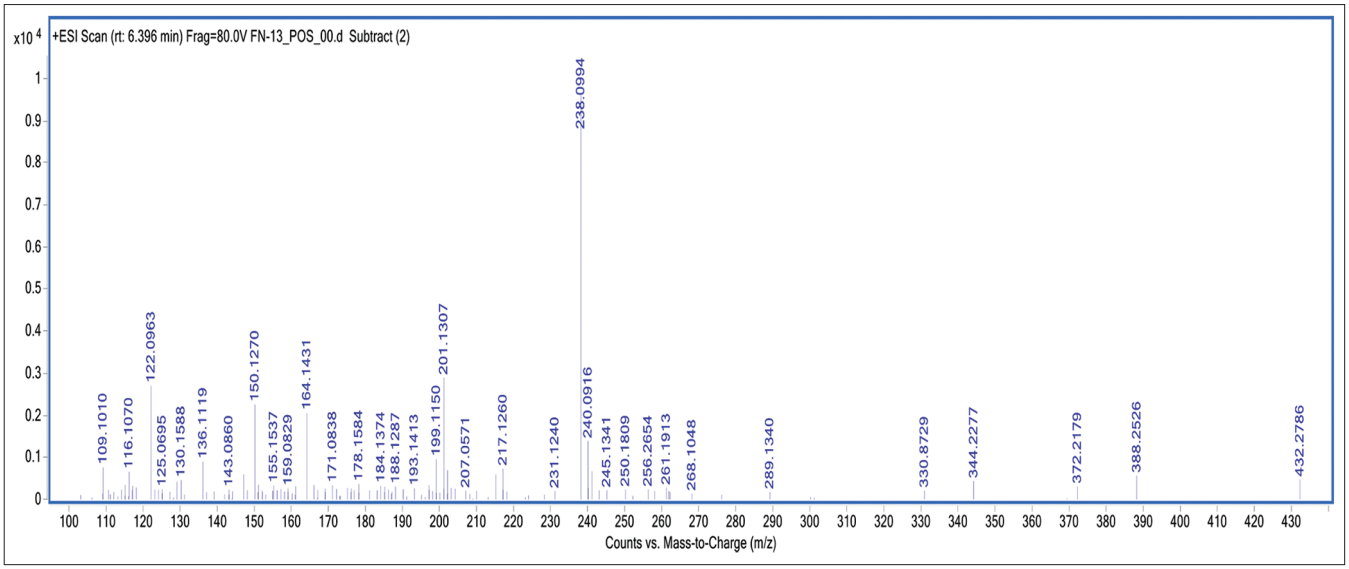


**Figure 10:** The mass spectra chromatogram of S3 (Fingernail-13) ketamine

**Table 9:** Comparative analysis of TN and FN data

| Analyte | No. of sample positive/total no. of samples tested | | Minimum–maximum concentration in ng/mg | |
|---|---|---|---|---|
| | FN | TN | FN | TN |
| Ketamine | 5/5 | 4/5 | 0.91–1.86 | 1.07–1.75 |
| Norketamine | 3/5 | 3/5 | 0.18–0.30 | 0.21–0.53 |

FN: Fingernail, TN: Toenail

**Table 10:** Earlier studies and their reported concentration ranges

| S. No. | Drug | Concentration range | Number of positive samples tested | Matrices | Method used | Reference |
|---|---|---|---|---|---|---|
| 1. | KT | 0.08–20.62 ng/mg | 7 out of 12 | FN and TN | UHPLC–MS-MS | Busardò *et al.*[13] |
| | NKT | (0.09–1.38 ng/mg) | | | | |
| 2. | KT | 0.04 ng/mg average in two positive cases | 2 out of 7 | FN and TN | UHPLC-MS/MS | Krumbiegel *et al.*[14] |
| 3. | KT | 0.52712ng/mg | 7 out of 9 | Nail clippings | UPLC-MS/MS | Mannocchi *et al.*[15] |
| | NKT | 0.19512 ng/mg | | | | |
| 4. | KT | <0.314 BLQ | 01 out of 7 | Nail clippings | GC-MS | Kim *et al.*[16] |
| 5. | NKT | <0.050 BLQ | | | | |
| 6. | KT | 3,772–12,632 | 122 in three-year period | Nail clippings | LC-MS/MS | Shu *et al.*[18] |
| | NKT | 201 | | | | |

FN: Fingernail, TN: Toenail, NKT: Norketamine, KET: Ketamine, BLQ: Below the limit of quantification, UHPLC-MS/MS: Ultra-performance liquid chromatography-tandem mass spectrometry, GC-MS: Gas chromatography-mass spectrometry, LC-MS: Liquid chromatography-mass spectrometry

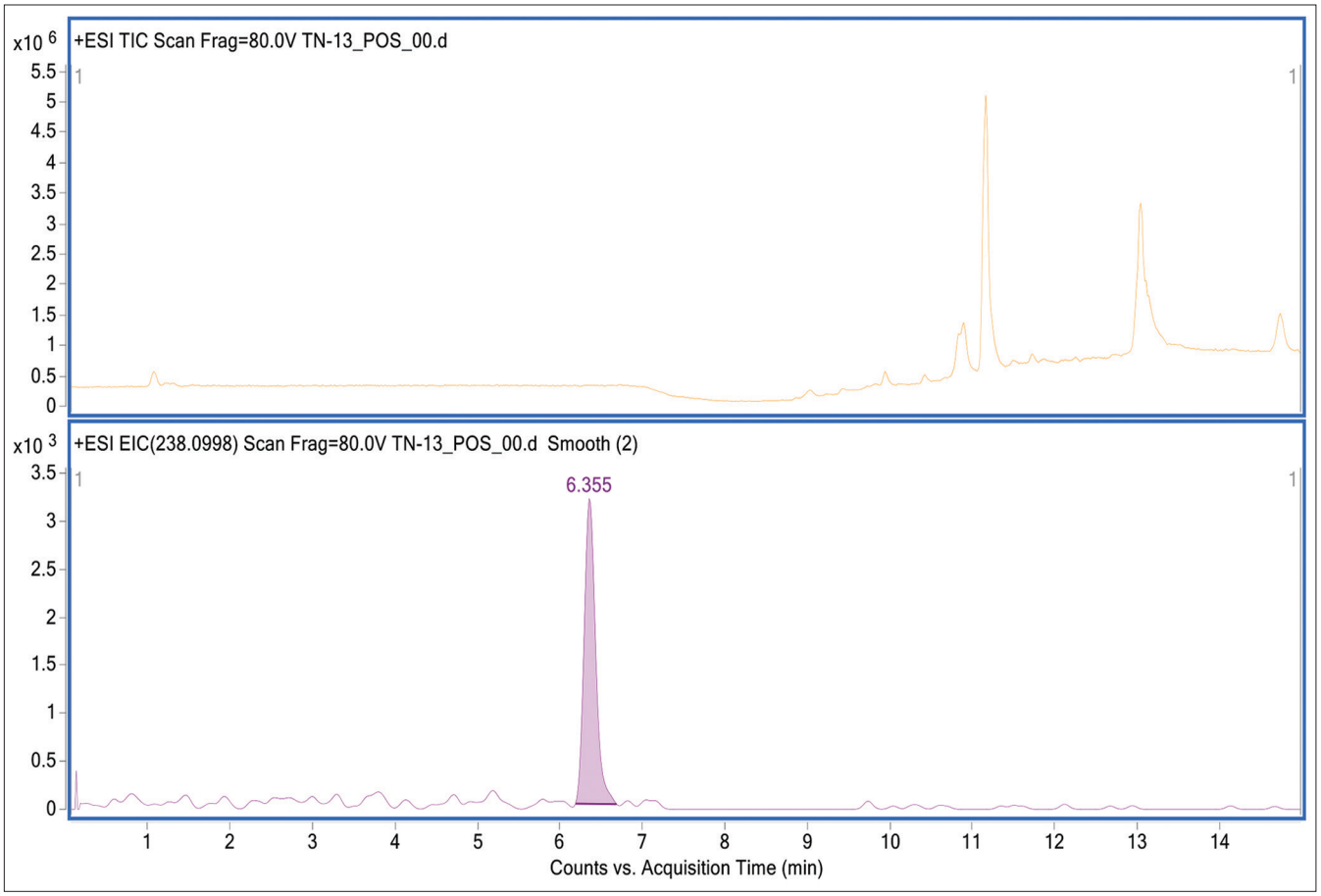


**Figure 11:** The chromatogram of S3 (Toenail-13) ketamine

There is still no published data specifically comparing the levels of KET and NKT in FNs and TNs. However, Busardò *et al.*[13] have compared the concentrations of these drugs, while Shu *et al.*[18] have conducted comparative studies on the concentrations of other types of drugs [Table 10], such as cannabis, alcohol and opioids in FN and TNs and they found that the concentrations of opioids at (51–118,229 pg/mg for FNs, 122–22,935 pg/mg for TNs), ethyl glucuronide

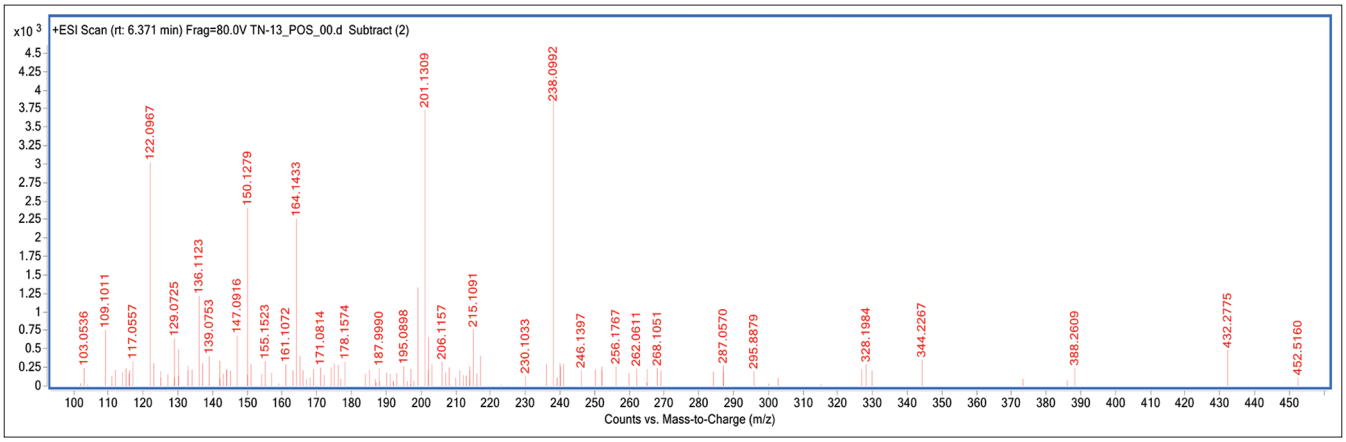


**Figure 12:** The mass spectra chromatogram of S3 (Toenail-13) ketamine

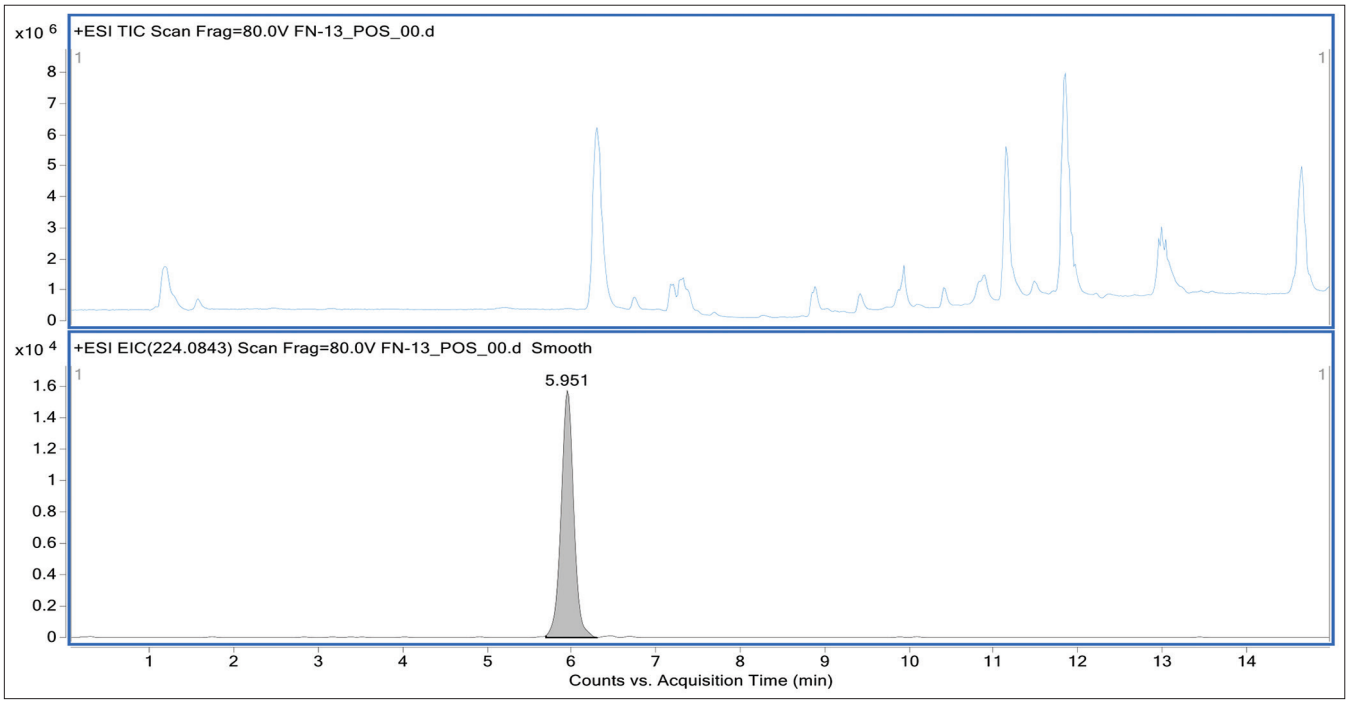


**Figure 13:** The chromatogram of S3 (Fingernail-13) norketamine

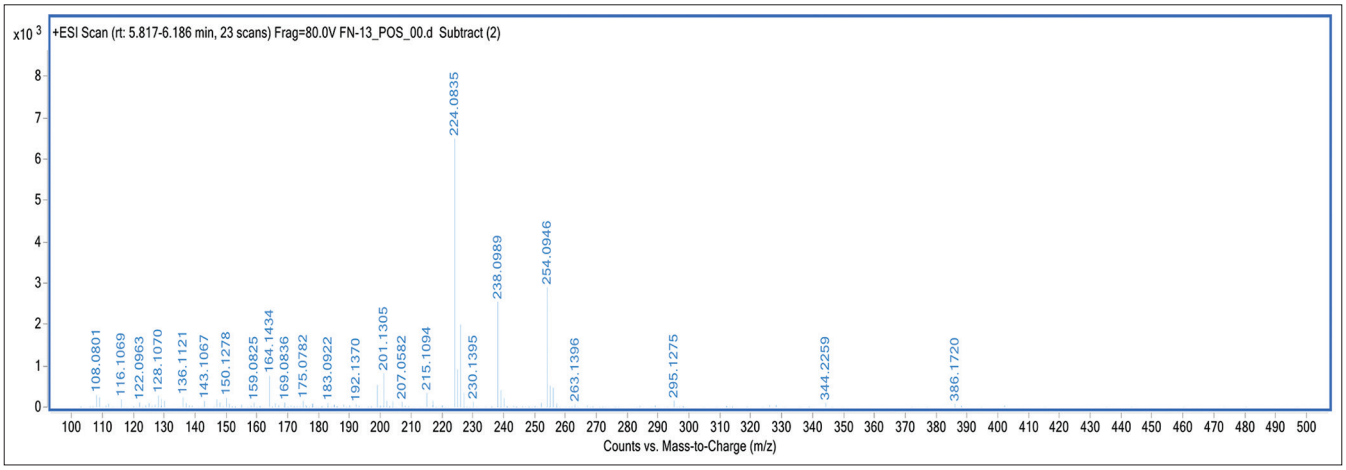


**Figure 14:** The mass spectra chromatogram of S3 (Fingernail-13) norketamine

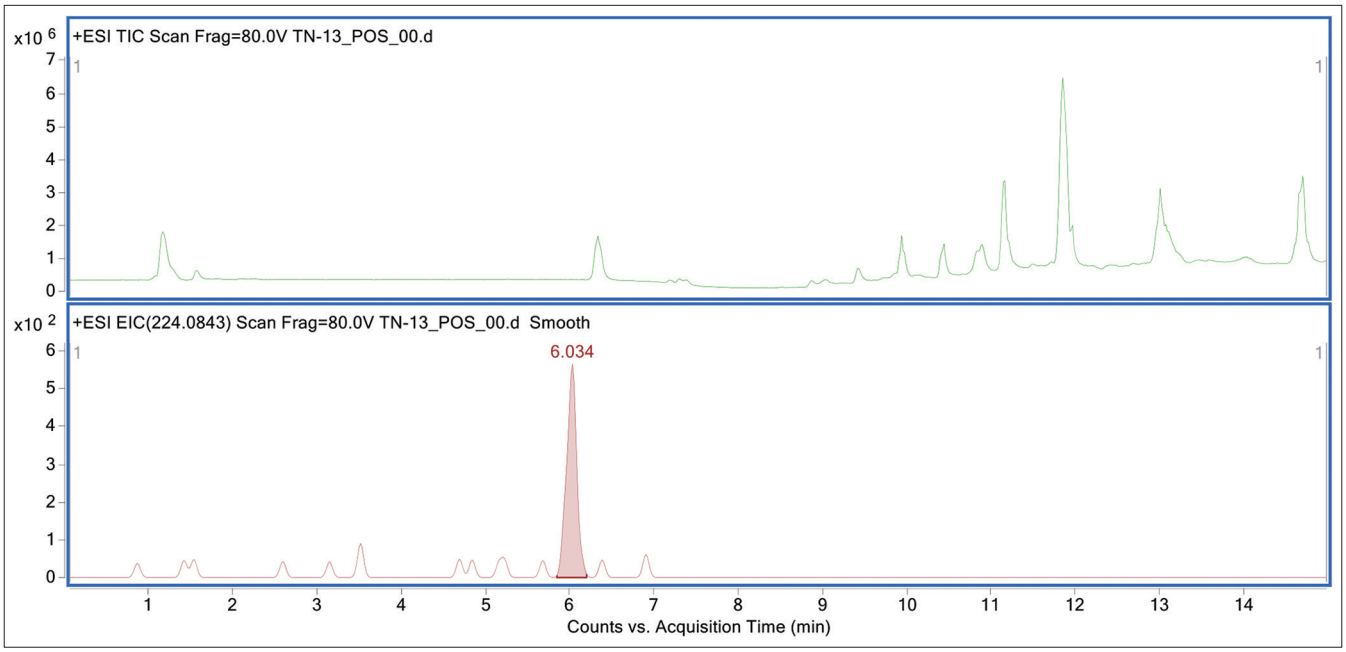


**Figure 15:** The chromatogram of S3 (Toenail-13) norketamine

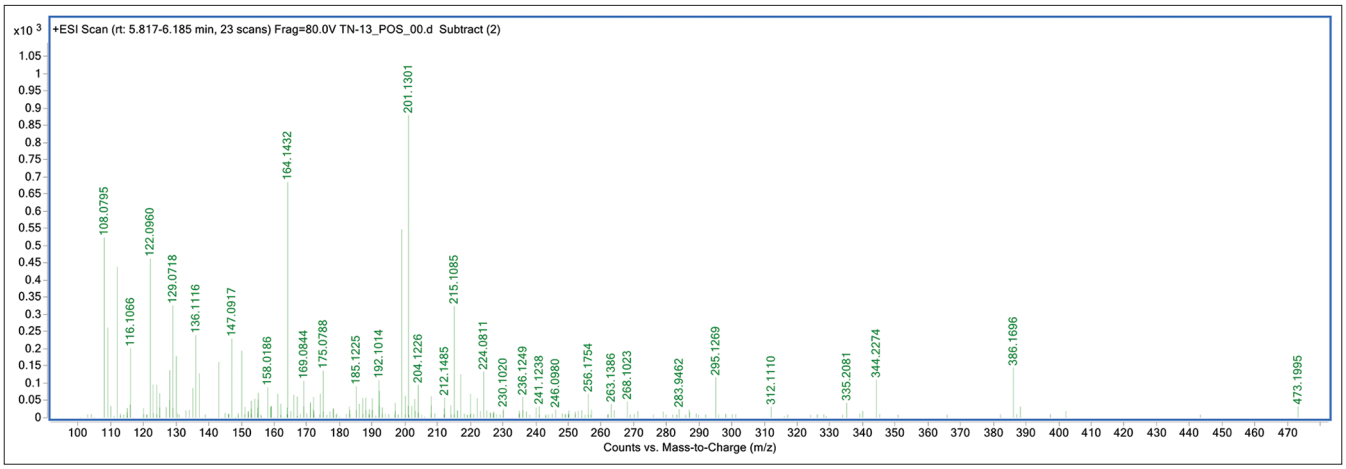


**Figure 16:** The mass spectra chromatogram of S3 (Toenail-13) norketamine

(20–3121 pg/mg for FNs, 23–254 pg/mg in TNs), Cannabis contents (0.12–146 pg/mg for FNs and 0.15–106 pg/mg for TNs) were significantly higher in FNs than TNs. A developed method specifically for KET and NKT is suggested which may be suitable in cases, such as drug consumption monitoring, drug-facilitated sexual assault, dacoity, and even in cases of medical negligence.

## CONCLUSION

A highly sensitive and rapid LC-QTOF-MS methodology has been developed and validated to ascertain the presence of KET and NKT in samples of FNs and TNs. This technique encompasses the processes of analyte identification, liquid-liquid extraction, and alkaline digestion. In comparison to conventional biological matrices such as blood and urine, the collection of nail and TN specimens is markedly less invasive and more convenient. FNs and TNs represent a viable alternative for the assessment of drug usage over extended periods, particularly when blood, urine, or hair samples are not obtainable, alongside the evaluation of prior drug consumption. The successful identification of KET and NKT in the FN and TN samples of individuals with a history of drug abuse was achieved utilizing this rigorously validated methodology.

## ACKNOWLEDGMENTS

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## ETHICAL APPROVAL

The Shri Vaishnav Vidyapeeth Ethics Committee affiliated with Shri Vaishnav Vidyapeeth Vishwavidyalaya, located in Indore, Madhya Pradesh, India, granted the requisite ethical approval under the reference code FDSR/2023/312, and formal consent was procured from the individuals enrolled in the rehabilitation facility.

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