DOI: 10.21088/jfct.2454.9363.11125.1

Journal of Forensic Chemistry and Toxicology
Volume 11 Number 1, January-June 2025

ORIGINAL ARTICLE

Simultaneous Determination and Method Validation For Opioids, Cannabinol and Nicotine in Postmortem Whole Blood Using High Performance thin Layer Chromatography Mass Spectrometry

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HOW TO CITE THIS ARTICLE:

Pallavi Choudhary, Kanak Lata Verma, Lijo T. Varghese et. al, Simultaneous Determination And Method Validation For Opioids, Cannabinol and Nicotine in Postmortem Whole Blood Using High -Performance thin Layer Chromatography–Mass Spectrometry. J Forensic Chemistry Toxicol. 2025; 11(1): 07–15.

ABSTRACT

Background: Opioids, cannabis and nicotine are the oldest and highly abused substances globally. The detection and identification of these drugs is a major challenge for the law enforcement agencies as well as forensic chemists, especially when the cases pertain to sexual assaults/drug-facilitated sexual assaults. Aim: To develop a determination method which can quantify opioids, cannabinol and nicotine simultaneously in whole blood.

Objective: This paper presents a validated method for the identification and quantitation of Morphine (MOR), Codeine (COD), Thebaine (THB), Papaverine (PAPA), Cannabinol (CBN) & nicotine (NIC) using High performance thin layer chromatography- Mass spectrometry HPTLC-MS, from whole blood at postmortem.

Material & Method: Quantitative analysis of MOR, COD, THB, PAPA, CBN and NIC were done using HPTLC and MS was operating in selective ion-monitoring mode for accurate identification of the drugs under study. Small Volume Liquid

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➢ **Received:** 15-05-2025 ➢ **Accepted:** 19-06-2025

extraction (SVLE) technique using ethyl acetate-hexane (80:20) were used for the extraction of blood. Pre-coated HPTLC (silica gel G 60 F254) plates were developed using mobile phase ethyl acetate: methanol: ammonia (8.5:1:0.5).

Result: The screening of HPTLC plates was done by UV light and the m/z ratio of drugs was obtained by lifting spots from plates using the HPTLC-MS interface. Method validation was done according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines. The technique will encourage forensic chemists to embrace the validated HPTLC-MS method.

Conclusion: The developed HPTLC-MS method is simple, sensitive, precise, accurate, economic and can be used for quantitative analysis of MOR, COD, THB, PAPA, NIC and CBN in blood.

KEYWORDS

• HPLTC-MS • Opioids • Nicotine • Cannabinol • Forensic Science

INTRODUCTION

Over the last few decades, the problem of drug abuse becomes a global phenomenon as more or less it affects each country with different magnitude. Illegal use of drugs affects the health of a person and produces morbidity and mortality to the individuals using them from direct actions. The abuse of drug is also instrumental in the society in terms of decrease in cultural values, increase in crime under the influence of drugs and also regulates the political grounds of a country.[1,2] Opioid, cannabis and nicotine are the oldest substance of abuse.[3,4,5] Natural opioids are products of opium plants. Cultivation and use of opium and its product are as older as human civilization. Morphine, codeine, thebaine, noscapine and, papaverine are the chief alkaloids of opium. Morphine, codeine, and thebaine belong to the phenanthrene group of alkaloids and scheduled under NDPS act in India. Morphine has been reported to be used as pain killer, codeine is used in cough syrups where as derivatives of thebaine are used in various medical preparation.[6] The occurrence of synthetic opioid fentanyl and its derivatives has grownsignificantly in forensic casework. Fentanyl has caused rapid increases in US and Canadian overdose deaths, yet its presence in illicit drugs is often unknown to consumers.[7,8] The ability of surface-enhanced Raman spectroscopy to detect trace amounts of opioids on clothing and packages has been investigated as the excessive use of prescription opioids and the use of very strong synthetic opioids, such as fentanyl, mixed with illicit street drugs has been reported in USA which is in the midst of an opioid crisis that included over 60,000 overdose fatalities in 2017.[9] Accurate laboratory analysis and drug identification will be critical in guiding individual medical management as well as gathering epidemiologic data to inform timely public health and law enforcement responses.[10] Like opium, cannabis is also abused worldwide in the form of charas, ganja, marijuana, hashish etc.[11]

The World Drug Report 2022 stated that nearly 284 million people of age group 15–64 used drugs globally in 2020, which was a 26% hike over the previous decade. In 2020, cannabis was the utmost common drug used by age group 15-64 years. 209 million people, which was 4% of the world's population, used cannabis worldwide in 2020. Opioid consumption continues to be extremely prevalent. In 2020, 61.3 million individuals (between a range of 36.5 million and 78.1 million) used opioids globally. This represents 1.2% of the world's population between the ages of 15-64 and includes both opiate and prescription opioid users.[12] A report (2019) published by National drug de-addiction treatment center, All India Institute of Medical Sciences, India in collaboration with Ministry of Social Justice and Empowerment, Government of India, stated that cannabis and opioids are the most common substance of abuse after alcohol in India. Nearly 3.1 millions of the Indian population, accounting for 2.8% used cannabis in any form or its product viz Bhang, Charas, Ganja. About 2.1% of the population of the nation use opioids and its product. Heroin is the most common opioid followed by pharmaceutical opioids and opium.[13] According to World Drug Report of

The United Nations Office on Drugs and Crime (UNODC) 2016, India is the cheapest market in terms of retail price of cannabis and opium.[14] Like tobacco, the smoking of cannabis has been associated with a diverse range of cancers and research has focused on the deleterious effects results of smoking cannabis which effects the functioning of human lungs, and lead to conditions such as chronic obstructive pulmonary disease.[15] India is vulnerable to drug abuse problem due to its geographical location as India is flanked bytwomain areas of illicit opium production of the world, the golden crescent i.e. Pakistan, Afghanistan, Iran and the golden triangle i.e. Vietnam, Laos, Thailand, Myanmar. The sandwiched position of India in between these areas makes it an appropriate route for transit for opium, cannabis amphetamines and synthetic drugs produced in these regions as well as fordrug trafficars.[16] Like opium and cannabis nicotine is also misused and overused worldwide. Tobacco smoke contains numerous hazardous components and contributes to serious adverse outcomes. Nicotine is toxic and highly addictive substance, it is important to develop and validate an easy and rapid analytical method to accurately measure nicotine level. Literature survey reveals that high performance liquid chromatography photodiode array detection (HPLC–PDA) method has been developed and validated for rapid determination of actual nicotine content in popular e-cigarettes liquids.[17] Gas chromatography-mass spectrometry (GC-MS) has been used for the identification of illicit drugs in vapes of e-cigarettes and for the identification of nicotine.[18]

Nearly six million people die every year worldwide due to abuse of tobacco and 1/6th of the total deaths occurs in India. According to statistics tobacco abuse causes one death in every six sec. The report of Indian council of medical research reveals that tobacco causes about 30% of all cancers in India.[19] Tobacco and tobacco related products are foremost threat factor for heart disease, stroke and bronchitis.[20] Considering these facts it is important to develop analytical methods for forensic identification of substance of abuse. The concept of hyphenating High-Performance Thin-Layer Chromatography with Mass Spectrometry has provided an incredible horizon for the detection of substances earlier believed to be challenging, especially pertaining to the biological exhibits in the forensic domain. TLC-MS Interface is a versatile instrument allowing rapid and contamination-free elution of TLC/HPTLC zones with direct transfer to the mass spectrometer and the presence of a substance can be confirmed via its mass spectrum or for an unknown substance, the respective sum formula can be obtained within a minute. The eluent is transferred directly to the mass spectrometer for its online analysis or the sample may also be collected for its offline analysis. The advantage of hyphenated techniques is that the separation and identification of known or unknown compounds is carried out in fraction of time period and the eluted zones may also be preserved for further investigation through spectroscopic techniques like Nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR). Quick and contamination-free elution of selected zones, direct transfer to the mass spectrometer, confirmation of known substances within a minute and low solvent consumption are the added advantage of using this technique.[16]

We have developed and validated the quantification method for morphine, codeine, thebaine, papaverine, cannabinol and nicotine using high performance thin layer chromatography-mass spectrometry from postmortem whole blood. On the basis of ICH guidelines[21], validation parameters such as Linearity, Accuracy, Precision, Recovery, Limit of Quantification and Limit of Detection were studied.

MATERIALS AND METHOD

Chemicals and Reagents: Standard of Morphine, Codeine, Thebaine, and Papaverine procured from Government Opium and Alkaloid Works, Neemuch, India, Nicotine and Cannbinol procured from Sigma-Aldrich. Ethyl acetate, n-hexane, Methanol, and Acetonitrile are of HPLC grade. Pre-coated HPTLC plates silica gel G 60 F254 procured from Merck India.

Preparation of Standard Stock Solution: 100µg/ml solution was prepared using Morphine (MOR), Codeine (COD), Thebaine (THB), Papaverine (PAPA), Cannabinol (CBN) and Nicotine (NIC) standard dissolved in methanol. Stock solution was filtered and sonicated for 3 min and stored at 4^0 C.

Preparation of Spiked Biological Samples:

The conventional approach to preparing a sample, referred to as liquid-liquid extraction (LLE), depends on partitioning an analyte between layers of two immiscible solvents, such as aqueous and organic. The primary downside of this technique is the extensive use of organic solvents and the numerous steps required to achieve the ideal analyte concentration.[22] To address the shortcomings of the LLE approach, the authors adopted small-volume liquid extraction in the current research paper. With this method, materials can be extracted in a single step using a very minimal amount of organic solvent in small centrifuge tubes.[23] Biological sample viz blood was extracted by Small Volume Liquid Extraction (SVLE) using Ethyl acetate-n Hexane (80:20) as extraction solvent.

Selection of Solvent Systems /Activation of Thin Layer Chromatographic Plates/ Saturation of TLC Developing Chamber: Various organic solvents in different ratios were used for selection of solvent system.[24]First of all TLC plates were washed with 20 ml of methanol then placed at 110^0 C for 30 min for activation. The developing chamber was saturated for 30min.After that HPTLC plates were developed in solvent system using Ethyl acetate: Methanol: Ammonia (8.5:1:0.5).

Sample application: Automatic sampler with 25 µl micro syringe was used for sample application of concentration 0.5ug-2.5ug per band (MOR), 0.5ug-2.5ug per band (COD), 0.25ug-1.5ug per band (THB), 0.1ug-0.5ug per band (PAPA), 0.1ug-0.5ug per band (NIC) & 0.02ug-0.2ug per band (CBN). Band length 6mm, plate size 10 x10. Methanol was used for sample application. Nitrogen was used as pressure gas. Plates were dried at room temperature for 10 minutes, starting position -8mm, solvent front position -80mm.

Development of plates: After sample application the plates were developed in the solvent system Ethyl acetate: Methanol: Ammonia (8.5:1:0.5). The development was done in10 x 10 twin through chamber saturated with mobile phase for 30min. Solvent front was moved to migration distance of 80mm of the plate. Plate was dried at room temperature.

Documentation: Chromatogram on the plates were visualized and photographed under UV 366nm and UV 254nm and in white light with 85 point exposure.

Scanning: After documentation TLC plates were scanned using TLC scanner 4 which was controlled by the software WINCATS. Under scanner spots were identified and position was marked by automatic scanning. Retention factor (Rf) of each compound was calculated. After proper marking of substance plate was again scanned under UV 200-400nm) using deuterium lamp. Spectra of each drug was recorded than analyzed.

Extraction of spots with TLC-MS interface (Camag): After generation of densitometry data of each standard TLC plate was subjected to TLC-MS interface for extraction of spot for MS analysis. With the help of UV light spot were marked on the plates. These marked spots were placed under the elution head of TLC-MS interface. Spots were lifted using methanol and acetonitrile as carrier solvent.These lifted spots, through mobile phase were introduced in the Shimadzu 2020 Mass spectrometer which was controlled by Lab solution software.

Experimental Condition of HPTLC/MS Table 1

Table 1: Instrument details and Chromatographic Conditions for High Performance Thin Layer Chromatography-Mass spectrometry (HPTLC-MS) Analysis

Instrument Details	
HPTLC	CAMAG
Mass spectrometer	MS 2020 SHIMADZU)
TLC-MS interface	CAMAG
Auto sampler	CAMAG ATS 4
TLC Scanner	CAMAG TS 4
Software HPTLC	WINCATS
Software MS	Lab Solutions
UV detection	254nm
Lambda Max	285nm
Mass parameters	
Mobile solvent for lifting up spot	Methanol (with 0.1% Ammonium Hydroxide)
Flow rate of mobile solvent for lifting up spot	0.5ml
Flow of Nebulizing gas	1.5L/min
Ionization mode	ESI with chemical ionization
DL temperature	2500C
Detector voltage	0.95KV
Mass Spectrum range (Scan Mode)	50-500 m/z

RESULTS AND DISCUSSION

High performance thin layer chromatography-Mass spectrometry is a hyphenated technique in which separationabilities of advance thin layer chromatography is combined with identificationabilities of mass spectroscopy

providing simple, rapid and highly sensitive method for identification and structural elucidation of various drugs. HPTLC is the developed version of TLC. Basic principle of separation is adsorption i.e. compound from the mixture to be separated is distributed between two immiscible solvents. Densitometry scanning is helpful for quantification by calculating Rf value and inspecting UV/Vis spectra of analyte (Figure 1 and 2) A mass spectrometer, is a combination of ionization, fragmentation and separation process which identifies the compound on the basis of their mass-to-charge ratio and produces a characteristic spectra of a molecule. Mass spectrometer is helpful in characterization and structural elucidation of analytes.[16,25] The aim of the present study is profoundly focused ondevelopment and validation ofa quantitative method for simultaneous determination of opioids, cannabinol and nicotine from postmortem blood sample. The spiked blood sample was extracted by small volume liquid-liquid extraction technique using Ethyl acetate-n Hexane (80:20). Plates were developed in the solvent system Ethyl acetate: Methanol: Ammonia (8.5:1:0.5). Developed HPTLC plates were scanned using CAMAG TLC Scanner and observations were recorded in terms of retention factor and maximum absorbance. The retention factor (Rf) value of MOR, COD, THB, PAPA, NIC and CBN was 0.14, 0.20, 0.25, 0.66, 0.36 and 0.83 respectively. The spot was lifted with CAMAG TLC-MS interface and injected into the mass spectrometer and the obtained mass spectrum were recorded (Figure 3 and 4). The analysis was done in both mode in MS, positive ion mode i.e. event 1 and other is negative ion mode i.e. event 2. The positive ion mode shows M-H at m/z 286 for Morphine, m/z300 for Codeine, m/z 312 for Thebaine, m/z340 for Papaverine, m/z163 for Nicotine and the negative ion mode shows major ions M-H at m/z309 for Cannabinol. The base peak present other than the actual molecular weight and the variation of ±1 is due to adduct formation of the drug sample with its solvent.[16]

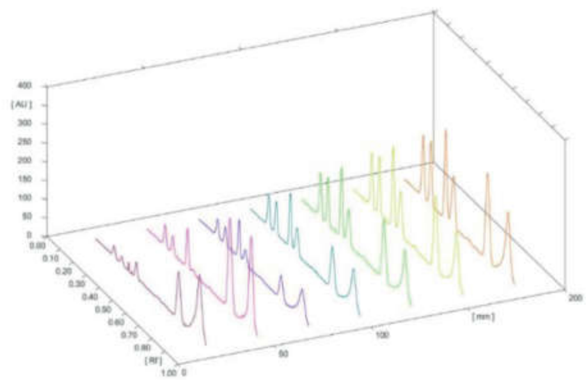
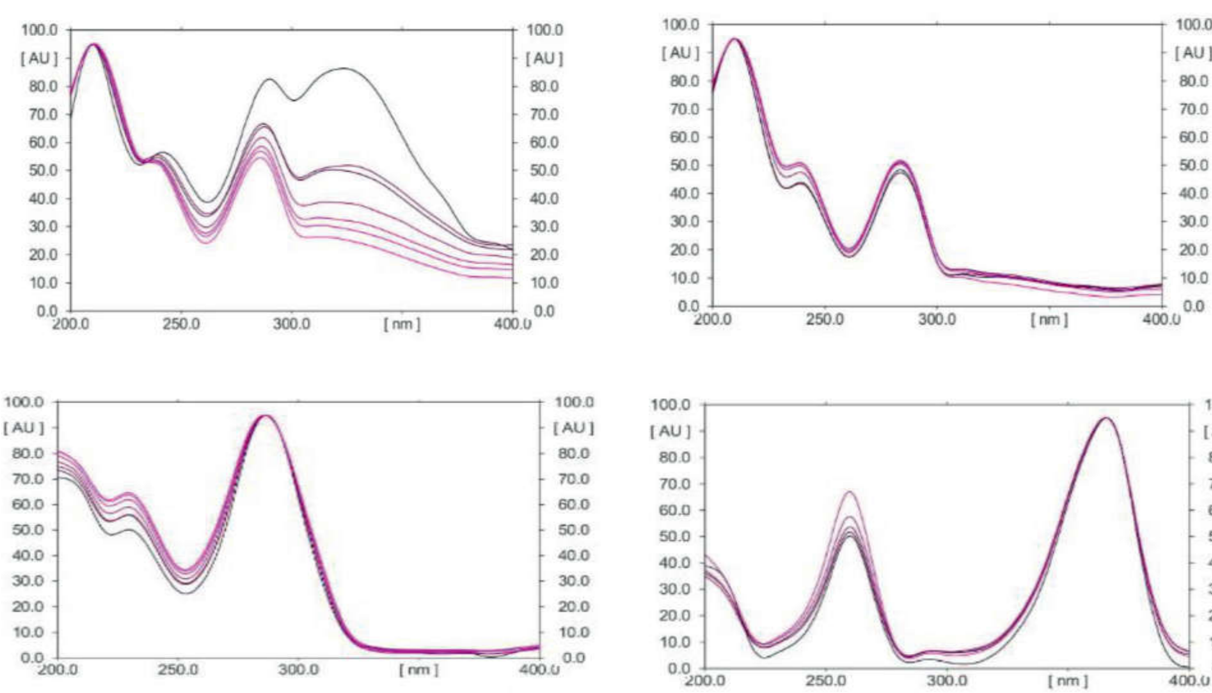


Figure 1: HPTLC Chromatogram of Morphine, Codeine, The baine, Papaverine, Nicotine and Cannabinol



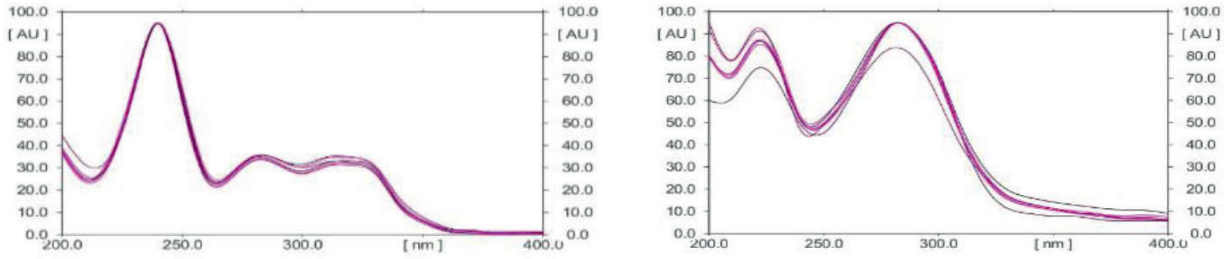


Figure 2: HPTLC Densitometric Chromatogram of **(A)** Morphine **(B)** Codeine **(C)** Thebaine **(D)** Papaverine **(E)** Nicotine and **(F)** Cannabinol

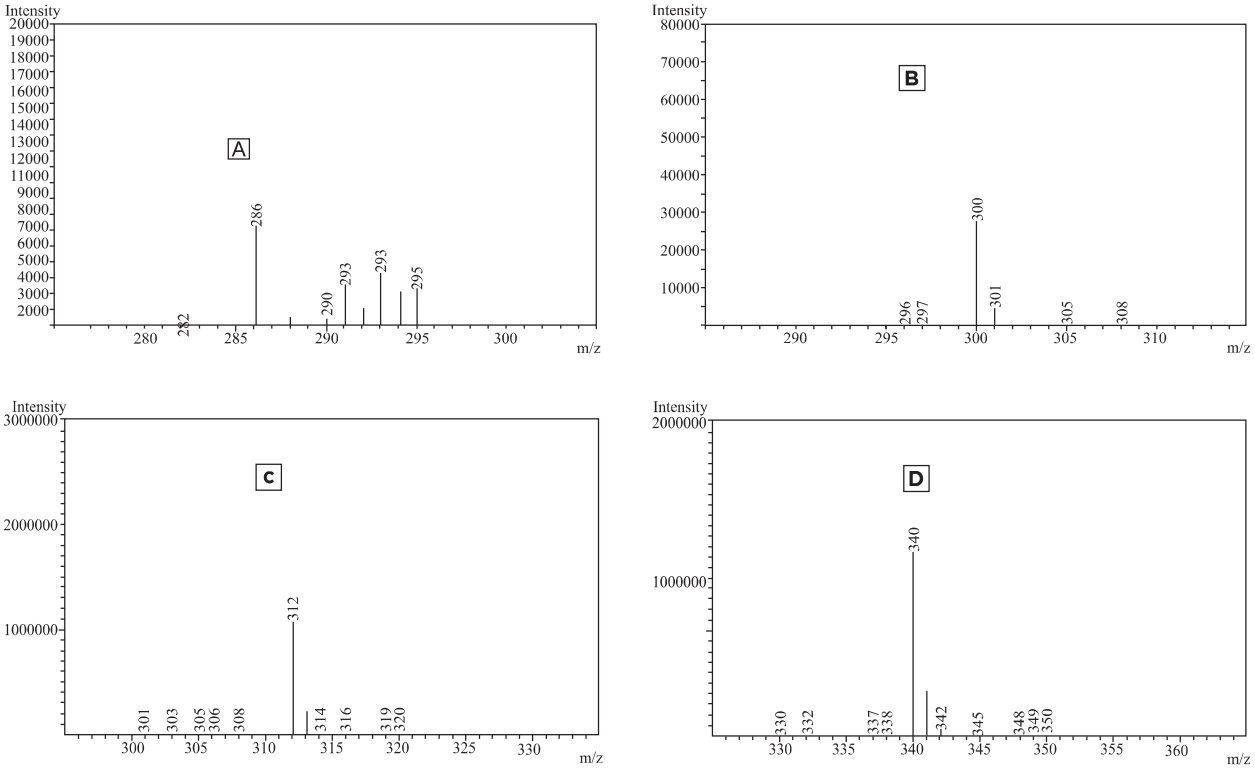


Figure 3: Mass Spectra showing m/z ratio of **(A)** Morphine **(B)** Codeine **(C)** Thebaine **(D)** Papaverine all in position ion mode

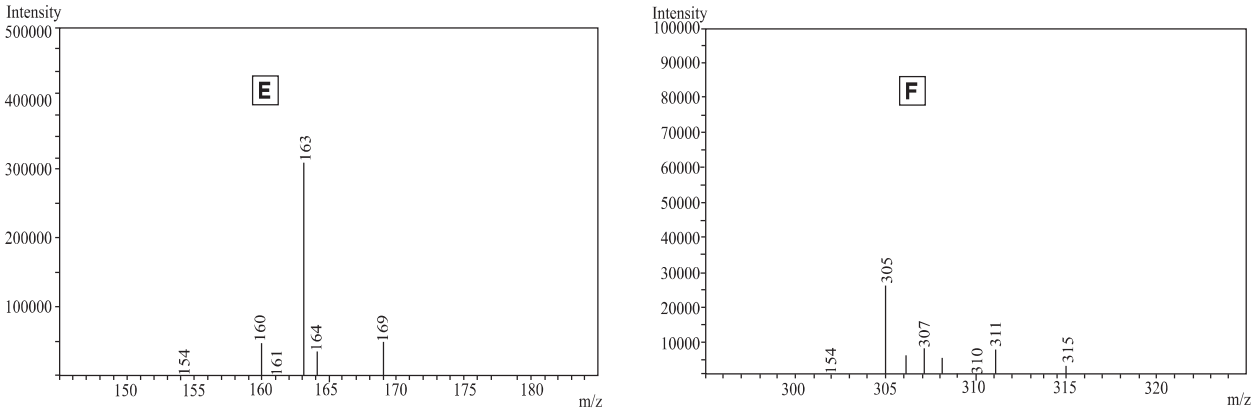


Figure 4: Mass Spectra showing m/z ratio of **(E)** Nicotine in positive ion mode and **(F)** Cannabinol in negative ion mode

Table 2: Method Validation Parameters according to ICH guidelines

Method Validation Parameters								
Compound	**Retention time**	**Linearity (ug/band)**	**Coefficient Correlation ±SD**	**Repeatability (RSD)**	**Precision**	**LOD& LOQ**	**m/z**	**Mean Recovery Percentage**
Morphine	0.22	0.5-2.5	0.999±1.96	1.94%	<5%	104.82ng 317.64ng	286 (+ve)	60-65%
Codeine	0.27	0.5-2.5	0.998±2.54	1.12%		132.23ng 400.70ng	300 (+ve)	
Thebaine	0.39	0.25-1.5	0.999±1.45	1.81%		45.74ng 138.61ng	312 (+ve)	
Papaverine	0.71	0.1-0.5	0.998±2.44	2.24%		23.78ng 72.06ng	340 (+ve)	
Nicotine	0.44	0.1-0.5	0.998±303	2.24%		59.74ng 181.05ng	163 (+ve)	
Cannabinol	0.87	0.02-0.2	0.998±2.53	1.41%		54.23ng 164.35ng	309 (-ve)	

The developed method was validated by studying specificity, Linearity, precision, accuracy, limit of detection, limit of quantification and recovery from blood samples according to the ICH guidelines (Table 2).[21] The data received was sufficient for separation, identification and quantification of each compound.The technique has encouraged us to move towards economic and clean technology and we are confident that this technique and our findings will encourage forensic scientists to embrace the new technology and contribute in reducing the burden on our environment.

CONCLUSION

In this present study the extraction of blood by SVLE technique was used as it takes less time and less solvent consumption compared with liquid-liquid extraction method. Quantitative analysis has been achieved by HPTLC and further confirmation has been achieved by lifting the spots by TLC-MS interface and m/z ratio determined the identification of substances. The base peak present other than the actual molecular weight and the variation of ±1 is due to adduct formation of the drug sample with its solvent. The technique shall encourage the forensic scientists embrace the economic and clean technology. The above developed HPTLC-MS method is simple, sensitive, precise and accurate and can be used for accurate quantitative analysis of morphine, codeine, the baine, papaverine, nicotine and cannabinol in biological samples such as blood. We are working on extraction of the drug from other body tissues as it is a challenge for forensic chemists to extract and detect these drugs whenthey present in traces,a problem to be addressed in forensic toxicology.Using the hyphenated techniques of HPTLC-MS, the separation and identification of these drugs can be carried out in fraction of time period. The eluent is transferred directly to the mass spectrometer for its online analysis and further the sample may be collected for its offline analysis. The eluted zones may also be preserved for investigation through spectroscopic techniques as well.

Declaration of Interest: None

Funding: This research did not receive any specific grant from funding agencies in the public, commercial or not-for profit sectors.

Highlights:

- Application of HPTLC–MS, an emerging hyphenated technique for accurate identification and quantification ofopioids, nicotine and cannabinol in biological samples submitted for forensic examination.

- Development and validation of method for quantification of morphine, codeine, thebaine, papaverine, cannabinol and nicotine using HPTLC-MS from postmortem whole blood.
- Contamination-free elution of selected zones, direct transfer to mass spectrometer, confirmation of known substances within minutes and low solvent consumption provides solution to address problems faced by forensic laboratories in exhibits examination pertaining to toxicological case work.

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