A REVIEW ON LIQUID CHROMATOGRAPHY-MASS SPECTROSCOPY: A MAJOR TOOL IN ANALYZING FORENSIC SAMPLES

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ABSTRACT

Forensic science deals with every aspect of science for the criminal justice system. Selection of instrument or techniques or even method should be critically judged to give accurate conclusions. In recent years, hyphenated techniques have been in trend for their advantage to perform a combined function. One such technique is LC-MS. These hyphenated techniques have proven to be a boon in the world of science. In the survey we found that new sample preparation techniques were utilized to concentrate the analyte present in the sample and to remove the interfering matrix element. This review article provides analytical data such as analyte under examination, sample preparation technique, percentage recovery, LOQ of analytes for biological samples of urine, blood, hair, plasma, serum, fetal postmortem brain in Forensic toxicology. The analyte under examination were drugs like cocaine, opiates, hallucinogens, amphetamine, cannabinoids, antipsychotics, and the tricyclic.

Key words: Liquid chromatography- Mass spectrometry, Forensic Toxicology, Sample Preparation, Drug of Abuse.

INTRODUCTION

Forensic science has been defined as the study and practice of applying science to the purpose of justice. Forensic science utilizes scientific principles to identify, interpret and search more for evidence materials. Technological advancement has increased use of more scientific and technical evidence in legal matters with respect to potentially toxic substances, their effects and their involvement in various criminal cases such answers can be provided by forensic technology. Liquid chromatography (LC or HPLC) is an important separation technique, especially for biomedical applications. In the field of forensic toxicology, the use of liquid chromatography has increased over the years. Approximately nearly 70% of everyday samples can be handled by LC in toxicology laboratory. However, overlapping of peaks and interference could hinder the simultaneous determination of drugs and its metabolites. Thermolabile, less volatile and polar analytes can be analyzed using LC. An upsurge can be seen in the utilization of LC coupled to mass spectrometry (MS) in the field of forensic toxicology, LC separates the analyte from matrix component whereas the mass spectrometer provides the mass to charge resolution of an analyte of interest. Over the years, sensitivity and resolution of instruments have improved and is a boon for bioanalytical applications. LC-MS do not require the analyte to be identified and quantified to undergo hydrolysis and derivatization procedures hence reducing the sample preparation steps. In spectra, LC-MS fragments only one ion in controlled environment hence are more robust. Various interfaces are available for LC-MS [1-3]. Forensic toxicology study of drugs employ blood, plasma or serum and urine for identification and quantification of the drug by single sample extract injection, hence saving time and resources. The compounds to be determined are mostly unknown, so the first step is to screen and identify any compounds of interest. Systematic toxicology analysis (STA) is a major part of the forensic study and is aimed at detecting and identifying all substances of toxicological relevance in biological materials [4]. Over the years, crimes committed due to the violation of drugs (such as

ethanol, cannabis, ecstasy, benzodiazepines and its analogs) has drastically increased, one of the reason being a better knowledge of side effects of drugs like hypnotics by people [5-8].

INSTRUMENTATION OVERVIEW OF LC-MS

Hyphenated techniques have been in trend due to their combined performances of two different analytical techniques, resulting in more specific and sensitive output. It is achieved by the coupling of two different analytical techniques by using proper interface. One such technique is LC-MS [9]. LC-MS combines the resolving ability of liquid chromatography and detection specificity of mass spectrometry, where LC separates the components in the mixture and MS detects charged ions. LC-MS is used for the analysis of thermally unstable, polar compound [10].

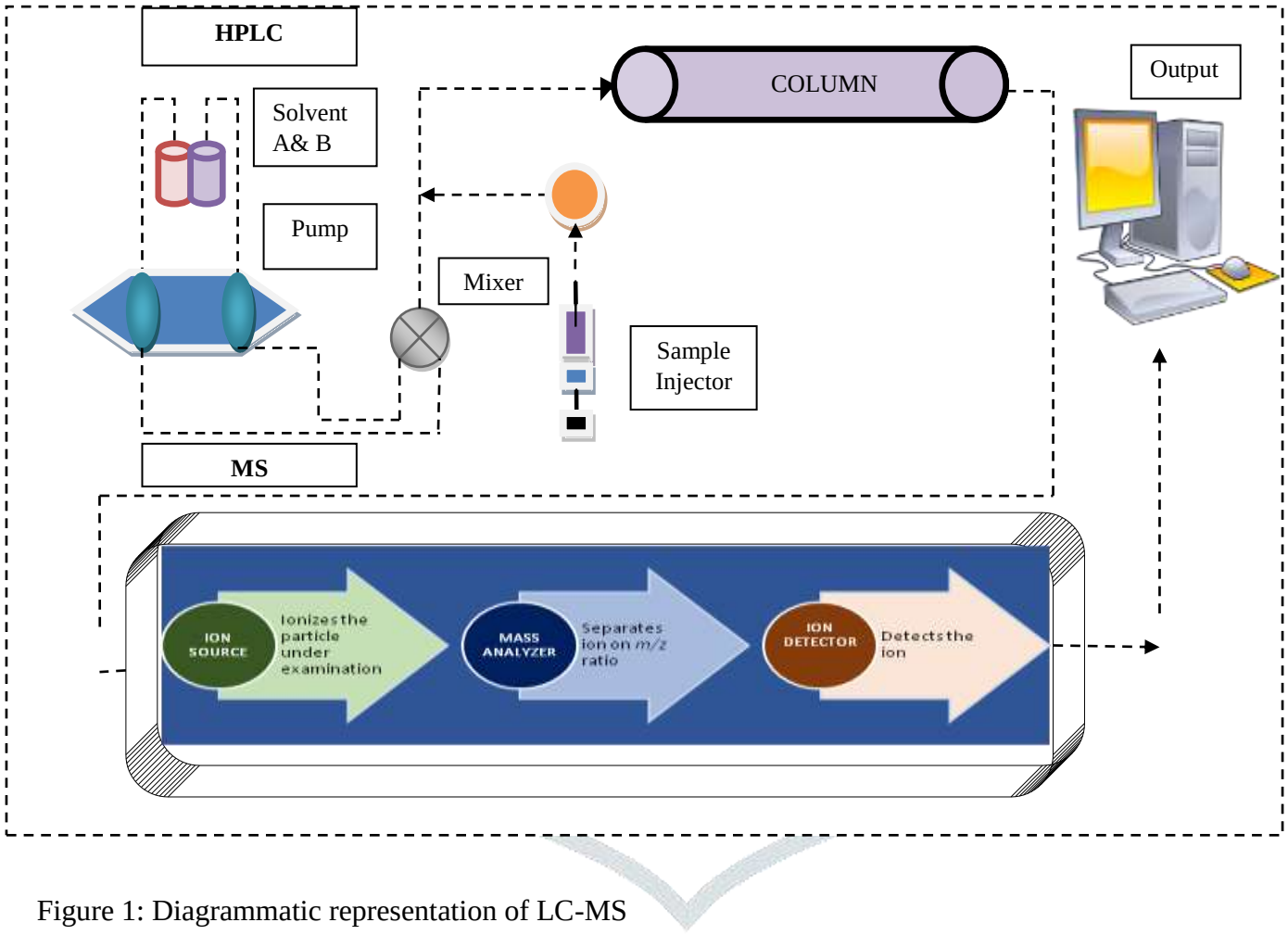

Figure 1: Diagrammatic representation of LC-MS

SAMPLE PREPARATION & FORENSIC TOXICOLOGY

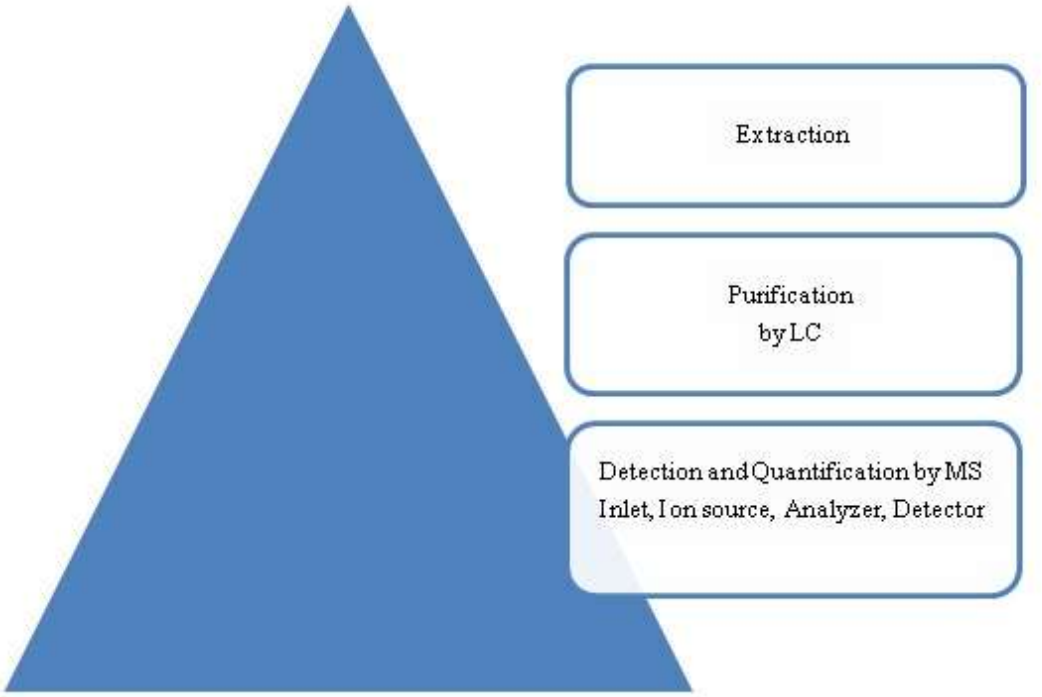

Figure 2: Diagrammatic representation of sample preparation by LC-MS

Biosamples and their Preparations

Biological sample preparation is an important part of biopharmaceutical analysis. An ideal sample preparation method should be cost-effective, environment-friendly, and involve fewer extraction steps. The goal of the sample preparation should be; concentrating the analyte at an appropriate level which increases sensitivity, removing the interfering matrix element (such as proteins, nucleic acids, sugars etc) that alter the MS response. Matrix effects in various ways such as ion suppression (loss of signal) or ion enhancement (gain in the signal) , or method variation leading to the negative impact on accuracy and precision. Clean sample results in better chromatographic separation with the lower limit of detection and decreased method variation hence are more robust. Due to the presence of analytes and their metabolites in the trace amount in matrices, sample preparation has become the more crucial step in forensic toxicology. In forensic toxicology field, the sample is prepared accordingly, whether the analyte is known or unknown. In case of known, the toxicologist aims to accurately determine the targeted analyte whereas in case of unknown, well-planned approach- systematic toxicological analysis is required [11]. A urine sample is primarily the sample of choice in case of the screening and identification of an unknown drug or poisons whereas blood, plasma or serum is the sample of choice in the case of targeted drugs or poisons [12-20]. Sample preparation requires isolation after cleavage of conjugates by enzymatic or acid hydrolysis (if required), and /or derivatization of the drugs and their metabolites. After isolation, the concentrated extract is dissolved in an appropriate volume of mobile phase [21]. Derivatization is not required for LC-MS, amines being an exception for poor chromatographic behavior [22].

Biological Sample Processing Techniques

In complex matrices, existing analytical techniques cannot directly determine the targeted analyte present in pictogram and nanogram levels. Biosamples undergo the process of isolation, purification, enrichment and the targeted analyte may often require chemical modification for detection. Thus, sample pretreatment plays a critical role. Traditionally, protein precipitation, centrifugation and separation, and LLE were widely used means of sample pretreatment. But due to many shortcomings, a new series of sample preparation techniques have arrived namely, solid phase extraction (SPE), solid phase microextraction technology (SPME), column

switching technology (CS), disposable pipette extraction (DPX), liquid phase microextraction (LPME) etc. These new techniques combined with LC-MS improve selectivity and chromatographic behavior [23-25].

Various Sample preparation techniques are discussed below:

SPE: It is a technique which separates compound in solutions by utilizing octadecyl (C-18) as stationary phase or more recently mixed mode phase with cation exchange. SPE column can elute several compounds and shows polar, non-polar and ionic effects. Eluents are then concentrated to dryness, diluted and injected into MS. SPE results are more accurate and precise with better recovery levels. SPE was first used for the extraction of the urine sample for determination of prostaglandin in human serum [26-29].

SPME: It is a more advanced version of SPE containing adsorbent on the fused silica surface. SPME is considered in the case of complex samples such as blood, plasma, tissues, whole blood, and urine. It is a separation technique in which stationary phase is coated on fiber and is placed on the solution. Analytes diffuse or are moved by convection into stationary phase. Then the extracted compounds on the fiber are desorbed and inserted into the injection port of a chromatograph. The advantage of SPME over SPE is that it requires a small amount of solvent and promotes endogenous and exogenous compound analysis [30].

DBS: In this technique blood sample is dried on the collection card, followed by the punching of the card and extraction of the target analyte from the card. The sample can be collected on either pure cotton filter paper or glass microfiber paper, where the rate of adsorption and dispersion depends on the thickness and density of the paper. For quantitative estimation of the analyte, blood should either be directly applied to the paper or via blow out method using capillary pipette containing a known volume of blood, then a fixed size disk (3-6mm) should be punched from the paper dried at 15º-22º C for the analysis. [31-32]

CS: It is a technique which is based on the selectivity of the stationary phases. This stationary phase retain and separates the analyte and eliminates the unretained components from the column. Another HPLC column is placed in series having different selectivity which enhances the separation of the targeted analyte. Once the targeted analyte is trapped in the inlet of the second column it is backwashed. This second HPLC column acts as the "injector" of the trapped components onto a third column, which affords the final analytical HPLC separation and elution to the LC/MS system. [33]

DPX: It is a technique in which "the solid phase sorbent is contained inside a disposable pipette tip and is mixed with sample solutions. This mixing allows less use of solid phase sorbent material which results in faster extractions. Elution can be performed using small amounts of solvent. Without the need for centrifugation or solvent evaporation, DPX methods can be readily be automated and the resultant eluents directly injected into liquid chromatography". [34-35]

SPMEM: In this technique sampling, extraction and concentration occur in a single step. This technique is the combination of both the solid phase micro-extraction (SPME) and membrane separation. The membrane is made up of polyethylene glycol (PEG), and polydimethylsiloxane (PDMS) as membrane material. PEG behaves as a pseudoliquid at the extraction stage. It is sufficiently robust and thermally stable. [36]

DLLME: It is a separation technique in which fine droplets are formed which are dispersed in the aqueous sample. These droplets are formed by the turbulence produced by the rapid injection of mixture containing organic solvent immiscible with water and a dispersive solvent miscible with water. This turbid mixture is then centrifuged and the fine droplets are sedimented at the bottom of the tube. A very high collective surface area of the droplets leads to the instant partitioning of analytes into the extraction phase. [37-38]

LPME: Based on hydrodynamic features, this technique can be classified into static LPME and dynamic LPME. In the static LPME, a solvent is used as an extractant and it is suspended in the sample. As a result transference of the target compounds to the extractant is carried out. On the other hand, in the dynamic mode, the exractant solvent forms a microfilm inside of an extraction unit, such as a microsyringe and the mass transfer of the analytes takes place between the sample and the microfilm. [39]

Molecularly imprinted polymers (MIP): It functions on the basis of recognizing specific properties of a molecule. MIP is stable even at high mechanical and thermal pressure. The solvent may sometimes impact

MIP. Compounds present in trace amounts in complex samples can be identified and quantified using this technique. One such derivative of MIP is MISPE in which extractions are automated. The process of concentration, separation and detection occurs by linking MISPE column to another instrument. In this precolumn is packed with MIP's particles before the column. The sample is loaded and the analytes are eluted by the mobile phase. [40-41]

ANALYTICAL DATA OF VARIOUS BIOLOGICAL SAMPLES

Sample preparation of urine in forensic toxicology

Different sample preparation techniques were applied to prepare urine sample for the detection of toxic analytes using LC-MS. Urine sample was investigated for various class of drug such as opiates, benzodiazepines, cocaine and its metabolites, hallucinogens, cannabinoids.

Table 1: Analytical data of various analytes in Urine samples

Class of Drug	**Analyte under Examination**	**Sample preparation Technique**	**Recovery (%)**	**Analytical technique**	**LOQ**	**Ref.**
Opiates	Morphine Codeine 6-AM Pholcodine Oxycodone Ethylmorphine	SPE	94-95% 93-95% 71-74%	UPLC-MS/MS	0.0079µg/ml 0.0070µg/ml 0.0032µg/ml 0.064g/ml 0.0050g/ml 0.0065g/ml	42
Benzodiazepines	Diazepam Oxazepam Nordiazepam Lorazepam	SPME	15-48	LC-MS/MS	0.2-2.0ng/ml	43
Benzodiazepines	7-aminoflunitrazepam	SPME	NA	LC-MS/MS	0.06ng/ml 0.034ng/ml	44
Benzidoazepines	7-aminoflunitrazepam	DLLME	NA	LC-ESI-MS/MS	0.08ng/ml	45
Cocaine & metabolites	BEG m-OH BEG p-OH BEG nor-BEG	SPE	NA	LC-MS/MS	1.2-5.0ng/ml	46
Hallucinogens	LSD 2-oxo-3-hydroxy LSD	SPE	>87	LC-MS	0.05ng/ml 1ng/ml	47
Hallucinogens	Ketamine	SPE	68-72	UPLC-	0.1ng/ml	48

	NK		61-65	MS/MS		
Hallucinogens	LSD	MISPE	83	LC-MS	0.2pg/ml	49
Hallucinogens	Dextromethorphan	CS	NA	LC-MS/MS	NA	50
Cannabinoid	THC CBD	SPMEM	NA	LC-MS	NA	51
Cannabinoids	THC-COOH	CS	100	LC-MS	5µg/l	52

Sample preparation of blood in forensic toxicology

Different sample preparation techniques were applied to prepare blood sample for the detection of toxic analytes using LC-MS. Blood sample was investigated for various class of drug such as opiates, benzodiazepines, hallucinogens, cannabinoids.

Table 2: Analytical data of various analytes in Blood samples

Class of Drug	**Analyte under Examination**	**Sample preparation Technique**	**Recovery (%)**	**Analytical technique**	**LOQ**	**Ref.**
Opiates	Methadone	SPE	NA	LC-MS/MS	0.009µg/ml	53
Benzodiazepines	Diazepam Oxazepam Nordiazepam Lorazepam	SPME	85-123	LC-MS/MS	4ng/ml	54
Benzodiazepines	Diazepam Oxazepam Nordiazepam	SPME *in vitro*	NA	LC-MS/MS	NA	55
Hallucinogens	Ketamine	SPE	NA	LC-MS/MS	NA	53
Hallucinogens	Dextromethorphan	DBS	87.8	LC-MS/MS	0.01ng/ml	56
Cannabinoids	THC THC-COOH	SPE	>85	LC-MS	0.25ng/ml	57

Cannabinoids	THC CBD	SPMEM	NA	LC-MS	NA	51
Cannabinoids	THC 11-OH-THC THC-COOH CBD CBN	CS	NA	LC-MS	1.8ng/ml 3.2ng/ml 2.8ng/ml 2.8ng/ml 7.7ng/ml	58
Cannabinoids	THC 11-OH-THC THC-COOH	CS	78 46 52	LC-MS/MS	0.44ng/ml 0.45ng/ml 2.00ng/ml	59

Sample preparation of hair in forensic toxicology

Different sample preparation techniques were applied to prepare hair sample for the detection of toxic analytes using LC-MS. Hair sample was investigated for various class of drug such as benzodiazepines, cocaine and its metabolites, hallucinogens.

Table 3: Analytical data of various analytes in Hair samples

Class of Drug	**Analyte under Examination**	**Sample preparation Technique**	**Recovery (%)**	**Analytical technique**	**LOQ**	**Ref.**
Benzodiazepine	Diazepam	MISPE	93	LC-MS/MS	0.14ng/mg	60
Benzodiazepine	8-benzodiazepine	MISPE	73-103	LC-MS/MS	0.11-1.32ng/mg	61
Cocaine and its metabolites	Cocaine BEG Cocaethylene Norcocaine	SPE	88.4-101.7	LC-MS/MS	50pg/mg	62
Cocaine and its metabolites	Cocaine and its metabolite	SPE	79.4-100.7	LC-MS/MS	0.05-0.34ng/mg	63

Cocaine and its metabolites	Cocaine and its metabolite	SPE	NA	LC-MS/MS	7-69ng/ml	64
Hallucinogens	LSD	MISPE	82	HPLC-MS	1pg/g	49
Hallucinogens	Ketamine NK	MISPE	86 88	LC-MS/MS	0.2ng/mg	65

Sample preparation of Plasma in forensic toxicology

Different sample preparation techniques were applied to prepare plasma sample for the detection of toxic analytes using LC-MS. Plasma sample was investigated for various class of drug such as hallucinogens, tricyclic antidepressants, antipsychotics.

Table 4: Analytical data of various analytes in Plasma samples

Class of Drug	**Analyte under Examination**	**Sample preparation Technique**	**Recovery (%)**	**Analytical technique**	**LOQ**	**Ref.**
Hallucinogens	Mescaline	SPE	72-84	LC-MS/MS	10ng/ml	66
Hallucinogens	Dextromethorphan	SPE	97.67-102.38	LC-MS/MS	5ng/ml	67
Hallucinogens	Dextromethorpha n	CS	NA	LC-MS/MS	NA	50
Hallucinogens	Dextromethorphan	CS	NA	LC-MS/MS	0.05ng/ml	68
Tricyclic Antidepressants	Desipramine Imipramine Nortriptyline Amitriptyline	SPME	1.21-7.67	LC-MS	50ng/ml	69
Tricyclic Antidepressants	Mitrazapine 8-OHM DMR	LPME	18.3-45.5	LC-MS/MS	1.25ng/ml	70

Antipsychotics	Quetiapine	SPE	>100	HPLC-MS/MS	1.0ng/ml	71

Sample preparation of serum in forensic toxicology

Different sample preparation techniques were applied to prepare serum sample for the detection of toxic analytes using LC-MS. Serum sample was investigated for various class of drug such as amphetamines, antipsychotics, hallucinogens.

Table 5: Analytical data of various analytes in Serum samples

Class of Drug	**Analyte under Examination**	**Sample preparation Technique**	**Recovery (%)**	**Analytical technique**	**LOQ**	**Ref.**
Amphetamines	AMP MAMP	SPME	6-8	LC-MS	0.9µg/l 0.13µg/l	72
Hallucinogens	PCP	SPE	57-66	LC-MS/MS	1ng/ml	73
Hallucinogens	Ketamine	SPE	89	LC-MS/MS	3ng/ml	74
Antipsychotics	Clozapine Clozapine-N-oxide Desmethylclozapine	SPE	>90	LC-MS	50.0ng/ml	75

Sample preparation of fetal post mortem brain in forensic toxicology

Different sample preparation techniques were applied to prepare fetal post mortem brain sample for the detection of toxic analytes using LC-MS. Fetal post mortem brain sample was investigated for opiates class of drug.

Table 6: Analytical data of various analytes in fetal post mortem brain samples

Class of Drug	**Analyte under Examination**	**Sample preparation Technique**	**Recovery (%)**	**Analytical technique**	**LOQ**	**Ref.**
Opiates	Morphine Codeine 6-AM	SPE	96.7-101.8 92.0-104.1 96.4-113.1	LC-MS/MS	5pg/ml	76

Sample preparation of oral fluid in forensic toxicology

Different sample preparation techniques were applied to prepare oral fluid sample for the detection of toxic analytes using LC-MS. Oral fluid was investigated for hallucinogens class of drug.

Table 7: Analytical data of various analytes in Oral fluids samples

Class of Drug	Analyte under Examination	Sample preparation Technique	Recovery (%)	Analytical technique	LOQ	Ref.
Hallucinogens	Ketamine PCP Mescaline	DPX	80-85 81-87 63-64	LC-MS/MS	1.5ng/ml 0.2ng/ml 0.2ng/ml	77

CONCLUSION

LC-MS proves to be a compelling tool in forensic toxicology applications. The key role of LC-MS in toxicology study is drug confirmation. LC-MS detects and quantifies more polar, thermolabile, and even drugs in trace amount. Sample preparation is a critical stage for the accurate finding of drugs in trace amount. In this review article, the prime focus is on the sample preparation techniques for LC-MS in forensic toxicology.

In conclusion, we have noticed in literature survey that primarily urine and blood have been used as the sample of choice for screening of drugs. Other samples which have been used are hair, plasma, serum etc.

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