Journal of Cardiovascular Disease Research

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 04, 2021

ESTIMATION OF NARCOTICS USING LC-MS/MS

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ABSTRACT:

Bioequivalence and bioavailability studies are essential in early (Pilot Bioequivalence) and late (Pivotal Bioequivalence) clinical development of drug candidates. Bioanalysis is the method to investigate the concentration of drugs, their metabolites and/or endogenous substances in the matrices such as blood, plasma, serum, cerebrospinal fluid, urine, and saliva. Chromatographic techniques like, HPLC, Gas chromatography, LC-MS, GC-MS, Ligand binding assay, immunological and microbiological procedures are used for the bioanalysis purpose. The method includes collection of sample, processing, storage in suitable conditions and finally analysis of a biological matrix for a drug. Sports drug testing laboratories are required to detect several classes of compounds that are prohibited at all times, which include anabolic agents, peptide hormones, growth factors, beta-2 agonists, hormones and metabolic modulators and diuretics. Other classes of compounds such as stimulants, narcotics, glucocorticoids are also prohibited, but only when an athlete is in competition. A single class of compounds can contain a large number of prohibited substances and all of the compounds should be detected by using testing procedure on LC-MS/MS instrument.

Key words:

LC-MS/MS; narcotics, biological matrix, bio fluids, bio availability

1. INTRODUCTION:

Bio-analytical methods are widely applied for the quantitative analysis of drugs, their metabolites and/or endogenous substances in biological matrices [1-3]. They play an important role in the evaluation of bioavailability, bioequivalence and pharmacokinetic studies [4-7]. The identification of newer analogues, the discovery of new drugs, a need for better methods of analysis that are less labor intensive, more accurate, more selective and changing regulatory guidelines are the driving factors for innovation in bio-analytical methods.[8-12].

Chromatographic techniques like, HPLC, gas chromatography, LC-MS, GC-MS, ligand binding assays, immunological and microbiological approaches are used for bio-analysis purpose. The documentation, validation and execution of these protocols are governed by ICH guidelines. The ICH M10 guidelines deal with bio-analytical method validation. The execution of these procedures is similar to other analytical procedures- sampling, sample pre-treatment/ preparation, loading, execution of analysis, data recording, data analysis and report generation [13-17].

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The bio-analysis of drugs and their corresponding metabolites in plasma and other biological matrices can be done by both HPLC and LCMS/MS methods. HPLC has been a dependable instrument for conducting bio-analytical protocols; however it is limited by the LOQ- limit of quantification of the available detectors. The electrochemical detector and the fluorescence detector are having greater sensitivity but the UV/PDA detectors are the most regularly used. The LCMS/MS methods are more sensitive, reliable and specific [18-21].

1.1 COLLECTION OF SAMPLE AND ITS PREPARATION [22–26]

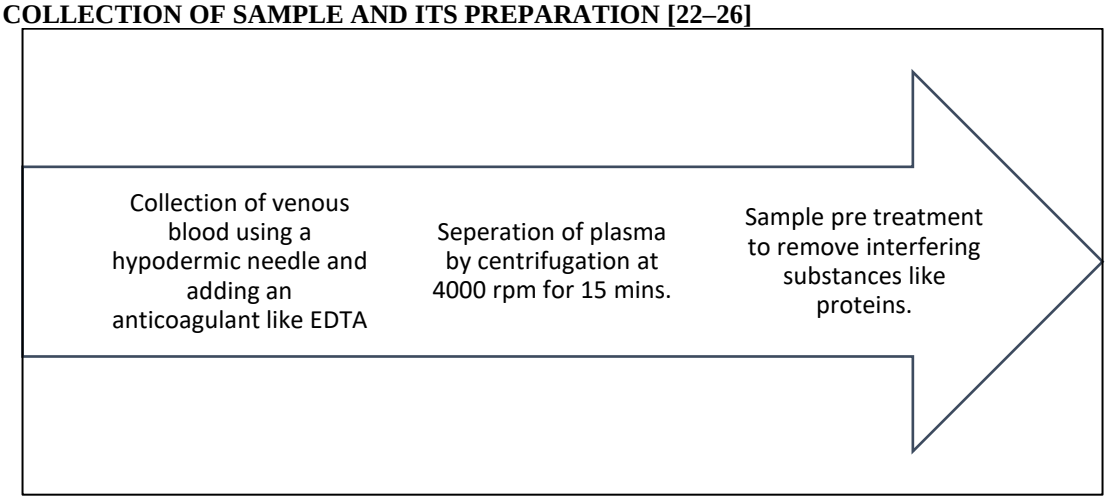

Fig. 1: Seperation of blood from plasma

1.2 BIOLOGICAL SAMPLE PREPARATION

Biological fluids are highly susceptible to physicochemical changes. Processing or purifying biological samples is often time consuming therefore optimal storage conditions must be established for biological samples. Samples sensitive to oxidation can be protected by using air tight containers. For moisture sensitive drugs dehydration could be achieved largely by freeze drying or lyophilization [27-30].

1.3 SAMPLE PRETREATMENT [30-32]

If the analyte is protein bound in such cases, protein precipitation may be done by altering pH, using organic solvents, acids and inorganic salts. If the analyte is not protein bound, the pretreatment may be done by centrifugation, homogenization, and hydrolysis of conjugates.

1.4 SEPARATION OF ANALYTE

Extraction procedures for analytes from biological samples may be done by one of the following procedures. These procedures are based on the principle of differential solubility of the analytes between aqueous and organic phases.

1. liquid-liquid extraction (LLE),
2. Solid-phase extraction (SPE)
3. Precipitation of plasma proteins (PP) [33-34].

Protein Precipitation (PP)

Protein precipitation is another important technique for extraction of the analyte from matrix. The principle behind PP is the precipitation (denaturation) of the proteins by using a range of reagents like organic acids (trichloroacetic acid and perchloric acid), organic solvents (methanol, acetone and acetonitrile) or by salts (ammonium sulphate). After denaturation, the sample is centrifuged, to remove the protein which settles at the bottom of the eppendorf tubes. [35-36]. This method may however clog the column.

Solid Phase Extraction (SPE)

SPE provides efficient recovery of the analytes and maximizes sensitivity of analysis. In this method the sample is passed through a small cartridge packed with adsorbent and the analyte maybe adsorbed onto the column or partitioned into the liquid phase. In case of adsorption the analyte is washed into a suitable solvent. In either case the sample is concentrated after extraction.[37-38] The column/cartridge is conditioned with the solvent of choice and the sample is loaded and washed to remove all other substances except the analyte. The analyte is eluted using a suitable mobile phase [39-40].

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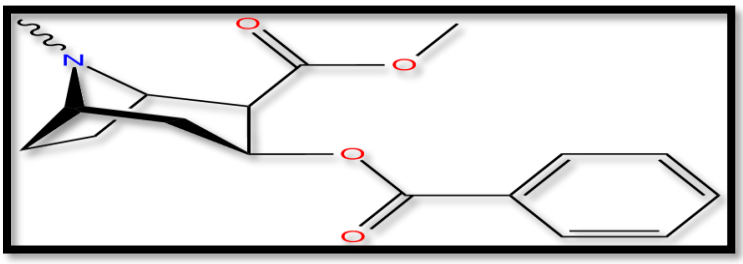
Table 1: **Types of Solid Phase Extraction Cartridges** [41]

Type of cartridge	Description
HLB Cartridge	Two monomers hydrophilic *N*-vinylpyrrolidone and lipophilic divinylbezene are present in specific ratio in HLB cartridge. It is available in various particle sizes such as (60ìm, 30ìm, 15ìm etc.)
MCX Cartridge	It is a mixed mode cation exchange, polymeric sorbent. It is a mixed-mode polymeric sorbent to achieve higher selectivity and sensitivity for extracting basic compounds with cation-exchange groups.
MAX Cartridge	MAX (mixed-mode anion-exchange) is usually intended to overcome the drawback of silica-based mixed-mode SPE sorbents. This cartridge has a mixed-mode anion-exchange, polymeric sorbent which is stable from pH 0 to 14.
WCX Cartridge	WCX (mixed-mode weak cation exchange) is usually intended to provide sample preparation for strong bases and quaternary amines. It has a water-wettable polymeric sorbent.
WAX Cartridge	WAX cartridge is for strong acids.WAX is mixed-mode weak anion-exchange reversed phase sorbent.
Bond Elute Plexa	It has non polar retention mechanism. It gives clean extracts which minimizes matrix interference.

1.5 NARCOTICS

The term narcotic has come to be associated with opioids, their derivatives and other habit forming drugs in common parlance. These are substances of abuse and as such their use has come to be regulated by corresponding government agencies all over the world to ensure safety [47-51].

COCAINE:



Cocaine is a tropane alkaloid with central nervous systems (CNS) stimulating and local anesthetic activity. Cocaine binds to the dopamine, serotonin, and norepinephrine transport proteins and inhibits the re-uptake of dopamine, serotonin, and norepinephrine into pre-synaptic neurons. This leads to an accumulation of the respective neurotransmitters in the synaptic cleft and may result in increased postsynaptic receptor activation. The mechanism of action through which cocaine exerts its local anesthetic effects is by binding to and blocking the voltage gated sodium channels in the neuronal cell membrane. By stabilizing neuronal membranes, cocaine inhibits the initiation and conduction of nerve impulses and produces a reversible loss of sensation [52].

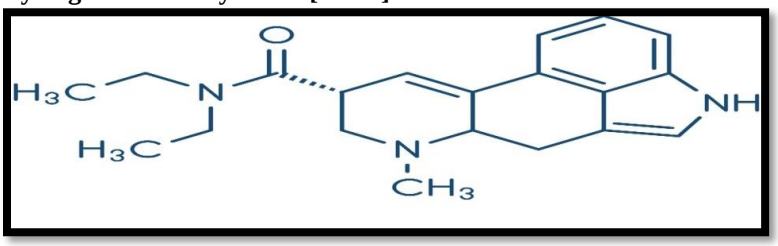
The sample extraction method discussed here was established in previous studies [53]. Briefly, internal standard (IS) solution (0.1 µM for each IS) with a volume equal to that of the whole blood was added to each blood

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 04, 2021

sample. The mixture was vortexed and then centrifuged for 15 min at 13,000 rpm; and the supernatant was collected and mixed with 500 µl of 4% formic acid. Before being loaded onto solid-phase extraction column, Oasis MCX 1 cc Vac Cartridge, conditioned by 1 ml methanol followed by 1 ml water, the sample was centrifuged at 13,000 rpm for 15 min. Loaded cartridge was washed twice with 1 ml methanol, and the contents were eluted twice with 500 µl methanol/water solution (95:5, v/v, with 7.5% ammonium hydroxide). Eluate was evaporated to dryness at 25°C using a vacuum concentrator, reconstituted in 74 µl 0.1% formic acid, and centrifuged at 13,000 rpm for 15 min. Supernatant was transferred to a vial and stored in a refrigerator until analysis by LC-MS/MS.[54].

Combined stock solution was prepared by mixing solutions of cocaine and its metabolites with those of the corresponding deuterium-labeled IS. The final concentration is 10 µM for each analyte, and 0.1 µM for each IS. Combined standard solutions were prepared by diluting the combined stock solution using the IS solution (0.1 µM for each IS) to various concentrations for each analyte and 0.1 µM for each corresponding IS. Calibration standards were prepared by adding 74 µl of different concentrations of combined standard solutions into 174 µl of blood mixture (74 µl whole blood + 100 µl paroxon solution) from untreated Sprague-Dawley rats. Quality control samples were prepared in the same manner. The same method as described above was used to extract cocaine, nine cocaine-related compounds, and all ISs.

Lysergic acid diethylamide[55-56]



LSD (lysergic acid diethylamide) was first synthesized in 1938 by Dr. Albert Hofmann, a natural products chemist at the Sandoz AG Pharmaceutical Company in Switzerland, as part of an exploration program that involved the systematic modification of the lysergamide scaffold. At that time, lysergamides were typically generated from lysergic acid purified from the ergot alkaloids produced by a parasitic rye fungus, *Claviceps purpurea*. The resulting lysergic acid would then be coupled with an alkylamine group (*e.g.*, diethylamine, dimethylamine, dipropylamine, etc.) to produce the amide. [57-58]

The HPLC system (Transcend TLX1 HPLC, Thermo Scientific, Basel, Switzerland) consisted of two Accela 1250 pumps for loading and eluting. The autosampler and sample extraction system were controlled by the Aria MX 2.1 software (Thermo Scientific, Basel, Switzerland). A cyclone P turboflow column (Thermo Scientific, Basel, Switzerland) was used for extraction, and a Zorbax Eclipse XDB-C8 column (Agilent, Santa Clara, CA, USA) was used for chromatographic separation. The online extraction system was coupled to a TSQ Endura triple stage mass spectrometer (Thermo Scientific, Basel, Switzerland) using APCI in positive mode because of its better performance with regard to matrix effects (24; 25).[59-60].

For LC, three mobile phases were used in gradient mode for extraction and analytical chromatography. Mobile phase A consisted of 20 mM ammonium acetate in water and 0.1% formic acid. Mobile phase B consisted of 20 mM ammonium acetate in methanol and acetonitrile (1:1) that contained 0.1% formic acid. Mobile phase C was an organic mixture of acetonitrile, acetone, and 2-propanol (1:1:1). Chromatography was run in isocratic mode with 70% mobile phase A and 30% mobile phase B, with a run time of 11 min and 4 additional minutes for flushing and equilibration using mobile phase C.

The positive ion discharge current was set to 5µA. The vaporizer temperature was optimized to 400°C. Sheath and auxiliary gas provided the best results, with flow rates of 15 and 5 arbitrary units, respectively. The temperature of the ion transfer tube was set to 300°C. The system was tuned and optimized for the detection of LSD. LSD and its metabolites were detected using SRM of the two to three most intense ion transitions [61-62].

HEROIN

Heroin is rapidly metabolized (figure 1) via 6-monoacetylmorphine (6-MAM) into morphine.Morphine itself is mainly metabolized into morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). The pharmacokinetics of the metabolism depends on the administration route [63]. Intravenous administration is the most common application route of heroin apart from inhalation. Basically, glucuronidation occurs in liver, catalyzed byUGT2B7 [64].

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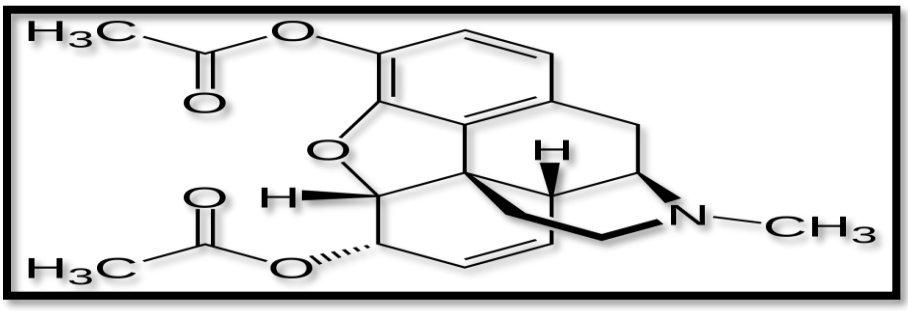

The extraction procedure is mainly based on the earlier described method [65]. For sample preparation 1 ml of serum was used. At first the internal standard (100 ng/ml of 6-MAM-d3 and morphine-d3 and 250 ng/ml of M3G-d3 and M6G-d3) and 2 ml of buffer solution (0.1 M ammonium acetate buffer; pH 9) were added. Afterwards, the samples were centrifuged for 8 minutes at 4000 rpm. For solid-phase extraction, Chromabond C18ec-SPE-columns from Macherey-Nagel GmbH & Co. KG (Dueren, Germany) were used. The columns were conditioned by 2 ml methanol, 2 ml of double distilled water and 2 ml of a buffer solution (0.1 M ammonium acetate buffer; pH 9). Subsequently, the samples were loaded onto the columns, followed by washing with 2 ml of buffer solution. Ahead of elution, the cannula was cleaned by 5 ml of bidistilled water and 1 ml of methanol. Analytes were eluted using 0.7 ml methanol followed by 0.7 ml methanol/acetic acid (9:1). The eluate was evaporated under a stream of nitrogen at 60 °C, reconstituted in 100 µl of HPLC mobile phase A and centrifuged for 10 minutes at 13,000 rpm. Samples were transferred into microvials and stored at -20 °C until analysis.[66-67].

The LC/MS/MS system consisted of a HPLC from Shimadzu coupled to a triple quadrupole mass spectrometer (AB Sciex 4000) using the positive ion mode. Due to the high polarity of the compounds of interest, a HILIC column (Nucleodur) from Macherey-Nagel GmbH & Co. KG, Dueren, Germany) was used for chromatographic separation. Mobile phase A (water, 15 mM ammonium acetate, pH = 4.3) and mobile phase B (pure acetonitrile) in a gradient program with a flow of 400 µl/min: 0-1 min: 95% B; 1-8 min 95% → 50% B; 8-10 min: 50% B → 10% B; 10-11 min: 10% B; 11-12 min: 10% B → 95% B; 12-15 min: 95% B. The transitions in multiple reaction monitoring (MRM) mode are listed in table 1 for all compounds. Since M3G and M6G are showing the same MRM transitions, good chromatographic separation is necessary [67-71].

1.6 CONCLUSION

The detailed method of analysis of different narcotics has been discussed above and given the habit forming proclivity of these substances there is constant need for further refinement of the analytical methods. Tandem mass spectrometry is an efficient and logical choice for the analysis of large quantities of samples in a relatively short period of time.

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