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LC-MS BASED BIOANALYTICAL TECHNIQUES FOR QUANTITATIVE ESTIMATION OF DRUGS RECENT ADVANCES AND REGULATORY PERSPECTIVES

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Abstract

Conventional bioanalytical techniques like, high-performance liquid chromatography with ultraviolet or fluorescence detection, have long been used for drug quantification. However, these approaches had historically faced various limitations in sensitivity, selectivity, and reliability when applied to the quantitative estimation of drugs in complex biological matrices. These challenges included inadequate assessment of trace level analytes, interference from endogenous matrix constituents, in addition these methods also struggle to differentiate structurally related compounds, including metabolites and isomers, resulting in compromised accuracy. Collectively, these challenges have posed significant obstacles in pharmacokinetic, bioavailability, and bioequivalence studies, where precise and reproducible measurements are critical. The introduction of liquid chromatography -mass spectroscopy (LC-MS) based bioanalytical techniques hassubstantially overcome these limitations. By integrating advanced chromatographic separation with high-sensitivity mass spectroscopy (LC-MS) enables the precise quantification of drugs and their metabolites even at minute levels. It offers high selectivity by identifying compounds based on their mass-to-charge ratio, which helps to reduce interference from other substances present in biological samples. This study is carried out to provide a comprehensive understanding of LC-MS based bioanalytical techniques that are used in quantitative estimation of drugs in biological matrices. Due to increasing demand for highly sensitive, selective, and rapid analytical methods in pharmaceutical and clinical research, LC-MS has become an essential and indispensable asset.

1. INTRODUCTION

Quantitative drug analysis in biological samples place a vital role in pharmacokinetics, toxicokinetic, therapeutic drug monitoring, bio-equivalency & metabolism studies. Among the various analytical techniques used in bio analysis; LC-MS have gained popularity over the last few years and have replaced HPLC in drug metabolism studies due to their high sensitivity and specificity. These techniques have potential to analyse bio fluids like; urine, plasma, serum & tissues.

1.1 What are Bioanalytical techniques?

Bioanalytical techniques are analytical techniques that are used for quantitative assessment of drug metabolites & biomarkers in living systems. They facilitate drug discovery and development as well as therapeutic monitoring of drugs efficacy and safety. Prior to introduction of LC-MS/MS, HPLC was predominant bio analytical technique and now has been replaced by more advanced techniques allowing for better performance of trace level measurements in complex matrices.

Recently, there has been a concern about the presence of residues from veterinary drugs in edible items that are obtained from animals, LC-MS has been used as a reliable technology to monitor and quantify these residues to ensure that animal derived food items are safe to enter the food chain. Using LC-MS technology, food industry can ensure that its products are meeting the standards ofregulatory requirements by providing confidence to the consumers in food safety [1]. LC-MS has wide range of applications in biological science, especially in pharmaceutical research like; drug discovery and development.

This advanced technology is used to study complex biological systems to help identified disease & quickly identified new drugs.

1.1.1 LC-MS technique

The pharmaceutical industry is under constant pressure to accelerate the development timelines. This demand is linked to a rise in number of biological samples that need pharmacokinetic analysis and decrease in desired quantitation

levels [2]. Hyphenated methodologies constitute some of the newly developed tools that are adopted by pharmaceutical industries to develop rapid and cost-effective analytical techniques.

One such highly popular methodology is LC-MS which has contributed significantly to quantitative bioanalytical research since 1990s due to its inherent specificity, sensitivity, and speed [2]. Liquid Chromatography coupled to Mass Spectroscopy is highly sophisticated evaluation techniquewhich plays major role in pharmaceutics for quantitativeestimation of drug and their metabolites in biological samples [1].

It combines separation abilities of LC with MS; spectroscopy identifies molecules that depends on mass to charge ratio. Whereas LC-MS provides a second dimension of separation based on fragment ions.

2. History of LC-MS [2]

2.1 Early development (1973-1988)

- First commercial LC-MS system has been introduced
- Scientists has developed new technique called Electrospray ionization (ESI) which converts liquid samples to charged particles.

Principle: Liquid sample is sprayed into tiny charged droplets; these droplets get evaporated and form gas phase ions. Previously it was difficult to combine liquid chromatography with mass spectroscopy but development of ESI (Electron Spray Ionisation) helped to combine both LC & MS.

-In this stage various other techniques like; Electron Ionization & Chemical Ionization was also developed.

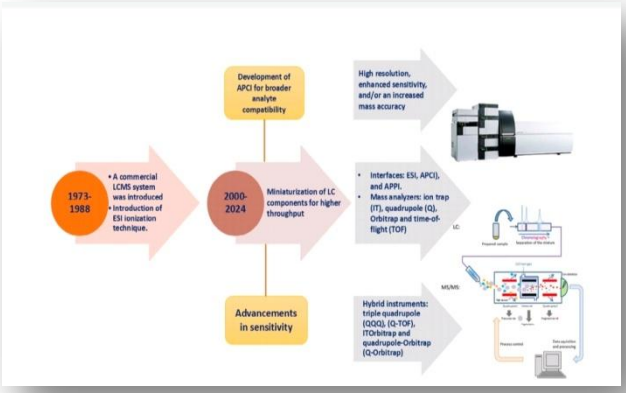

Figure 01 Progress in LC-MS development in last few decades. [2]

2.2Mid era (2000-2010)

2.2.1 Development of DESI (Desorption Electrospray Ionization)

- It is developed to eliminate some limitations of traditional ionization methods to enable fast, direct, and ambient analysis of samples with minimal preparation.

Principle: when the spray hits on surface it removes the molecules and ionizes.

2.2.2 Development of APCI (Atmospheric Pressure Chemical Ionization).

Since some drugs cannot be analysed by ESI, this development is used for analysis of low polarmolecules and moderately volatile substances.

Principle

liquid sample is sprayed into a heated gas stream where it forms an aerosol and get evaporated leaving gas phase analyte molecules these ions are charged by ion transfer by a high-voltage corona needle and are detected by the MS.

2.2.3. Miniaturization of LC-MS components.

- Miniaturization is the process of making things smaller and more efficient.
- It improved smaller columns, faster pumps, better detectors.
- By this; more samples are analysed in less time with less solvent consumption.

2.3Advancements (Later era) 2010-2024 [3]

The continuous improvement of instrumentation is the key to LC-MS success **[4].** LC systems have evolved from basic manual pumps and columns to sophisticated automated systems that provide precise control over chromatographic separations**.**

2.3.1Increase in sensitivity and accuracy.

New LC-MS technology offers;

a. High resolution – Better separation of compounds.
b. High sensitivity – Can detect low concentration of drug.
c. High accuracy – Can identify exact molecules.

It is very useful in; pharmacokinetic studies, bioequivalence studies, drug metabolism studies & therapeutic monitoring.

2.3.2 Mass Analysers (High precision systems)**.**

Mass analysers measure the mass to charge ratio of ions. They are;

a. Ion trap (IT)
b. Quadrupole (Q)
c. Orbitrap
d. Time of flight (TOF)

Each of these analysers has its own advantages in terms of speed, sensitivity & resolution.

Ion trap (IT): A device that uses electric fields to capture and hold ions in a confined space (either 3D or 2D linear trap). Once trapped these ions can be manipulated, fragmented multiple times and then ejected sequentially to be measured.

Quadrupole (Q): A mass filter consisting of four parallel cylindrical rods. By applying specific oscillating radiofrequency and DC voltages, it only allows ions with a specific mass-to-charge ratio to pass through the centre to the detector, while others crash into the rods.

Orbitrap: It is a high-resolution analyser where ions are injected into an electrostatic filed &forced to orbit around a central, spindle-shaped electrode. The frequency of their axial oscillations is measured and converted into highly accurate mass data using a Fourier Transformer.

Time-of-Flight: It is an analyser; itassessesthe timefor ions to travel a fixed distance through a vacuumtube. Since all ions are given the sae initial kinetic energy, lighter ions will travelfast& than that of heavier ones and reach the detector

2.3.3 Development of Hybrid Instruments.

The new technology combines two analysers in one instrument.

Types of combined instruments are;

- Triple quadrupole (QQQ)

- IMS (Ion Mobility Separation).
- These instruments offer better quantifications & structural identification of drugs.

Triple Quadrupole (QQQ): Triple quadrupole is a type of mass spectrometer, which has three quadrupoles in series, where the first quadrupole (Q1) is used for selecting ions, second quadrupole(Q2) is used for fragmenting ions, and Q3 is used for fragment analysis.

Ion Mobility Separation (IMS): It is a technique which separates the ions based on their shape, size& charge while they are moving in a gas.

3. Working of LC-MS. [5]

1. Sample preparation (plasma, urine etc.
2. Separation - Using LCMS i.e., mixture of drugs is separated.
3. Ionization - Drug molecules are converted into ions using ESI/APCI/APPI.
4. Mass analysis – MS determines the mass to charge ratio
5. Detection & Data processing – The computer generates chromatograms and spectra to identify and quantify the drugs.
6. Calibration.
7. Validation.

3.1Samplepreparation in LC-MS. [6]

Satisfactory sample preparation is a key aspect of measurement of drugs; sample preparation is the only process which slows down the work during analysis.

Sample preparation in LC-MS is mainly of 2 methods;

3.1.1 Off-line sample preparation.

3.1.2 Online solid-phase extraction.

3.1.1 Off-line sample preparation: Automated off-line preparation refers to the process where the sample preparation is done outside the instrument i.e., once after the sample is prepared it is then injected into the instrument.

It involves various other techniques like;

a. Liquid-Liquid extraction (LLE)
b. Solid-Phase extraction (SPE)
c. Protein precipitation (PPT)

(a)Liquid-liquid extraction: This method provides high-quality sample clean up but faces engineering difficulties particularly when trying to automate high-throughput processing. Since it requires mixing two liquids and waiting for phase separation, it is time-consuming and demands high precision **[2, 7].**

Common extraction solvents such as methyl t-butyl ether (MTBE) or ethyl acetate are used in routine extraction of plasma, blood, or tissue samples **[2, 8].**

Eg; Wang et al. have developed & validated a 96-well LLE assay, using LC-MS/MS in APCI mode for simultaneous quantification of Lopinavir &Ritonavir [**2, 9].**

(b) Solid-Phase extraction [2, 7]: This method utilizes various sorbents that are either silica based (or) polymer based (or) Mixed-mode polymer-based sorbents (e.g., Waters Oasis MCX cartridge) for isolation of biological fluids.

SPE gives superior results when compared to PPT (protein precipitation) but is of higher cost due to labour & material cost.

(c)Protein precipitation [2, 6]: Sample preparation with protein precipitation is widely used and rapid preparation technique for bioanalysis.

The method has been extended to measuring of drug & metabolites from whole blood.

E.g., Koseki et at. have developed a sensitive & specific LC-MS/MS method for the simultaneous determination of cyclosporine A and its three metabolites AM1, AM4N, &AM9 **[2, 7].**

Protein precipitation offers a fast sample preparation technique that is easily automated. However, salts & endogenous materials are still present when analysing supernatant from plasma & cause ion suppression that led to larger variations.

Later; **Combination of sample preparation** methods have been developed to achieve higher purity &throughput simultaneously.

E.g., Xue et al. investigated a simplified PPT / mixed-mode cation-exchange SPE procedure.

Procedure: Acetonitrile & methanol along with formic acid was used to precipitate plasma proteins. AfterInteraction &centrifugation, the supernatants are directly loaded onto a 96-welled extraction plate, where the drug was retains at sorbent that is negatively charged, while interfering endogenous materials were eliminated.

3.1.2 Online solid-phase extraction: The online SPE offers high sensitivity&speed byintegrating pre-concentration factor&low extraction cost.But it uses program-controlled switch valves & column re-configurations [2].

Online technique will be completelyautomated. Most online approaches of solid phase extraction use column-switching to couple with analytical columns.

There are various extraction supports which allow direct injection of biological samples. These supports include Restricted Access Media (RAM), large-sized particle, monolithic material& disposable cartridges.

E.g., A polar functionalized polymer has been explored as extraction support in this method [2].

3.2 Separation [5].

Biological samples are complex matrices containing many interfering substances.

LC-MS separation is necessary due to matrix effects improving quantitation, isobaric compounds, isotopic interference, and chemical interconversions.

LC -MS separation step can be further enhanced using various approaches like: UPHLC- Ultra Performance High Liquid Chromatography, Core-shell columns, Monolithic columns, HTLC- High-performance Thin Layer Chromatography.

Procedure: initially mixture of sample is injected into the LC-MS system which contains the mobile phase (mobile phase used in LC-MS are of low cost, UV transparent, low viscosity, non-corrosive to LC system component). This mobile phase takes the sample in to the column where the actual separation happens. (columns used in LC-MS are; (a) short column (15-50mm), (b) large column (20-250mm).

3.3 Detection [5].

- In case of detection the separated compounds are identified and measured after coming out of chromatographic column.
- The main requirements for bioanalytical detection are: highest level of sensitivity, selectivity, and wide linear range of the samples.

- ESI (electron spray isolation) is the isolation method that is used for detection of polarmolecules.
- Triple Quadrupole is most widely used for quantitative bioanalysis but has low resolution.

HRMS (high-resolution mass spectroscopy) is used in both the targeted and non-targeted analysis. It is suitable for screening of multiple analytes and is less sensitive when compared to triple quadrupole.

3.4Calibration

The calibration method has become software driven and hence peak integration is no longer manual but handled by software systems, hence human errors are reduced.

Calibration can now include detailed data verification steps, like; retention time, consistency, peak shape but not just signal, measurements.

Calibration in case of LC-MS is the process of ensuring that the instrument gives accurate and reliable results when measuring compounds.

Procedure: Standard solutions of unknown concentrations are prepared and injected to LC-MS and the response is generated, signal should be recorded for the response and plot the calibration curve and validate it now use the curve to calculate the unknown concentrations.

3.5Method Validation [5]

Method validation is now performed based on widely accepted guidelines such as ICH.

The method is carried out to confirm whether the analytical technique is suitable for its designed purpose.

The method is evaluated by checking the key performance parameters including selectivity, sensitivity, linearity, accuracy.

The parameters are tested at different concentration levels, usually to ensure consistency.

The method also includes stability studies to know the analyte performance under various conditions to ensure reliable performance overtime.

4.Present Scope.

LC-MS is widely applied for the rapid and variable screening of drugs and their metabolites in biological samples **[8-10]**

It enables simultaneous detection of multiple compounds, making it highly suitable for multianalyte analysis.

The technique is extensively used in toxicology for the identification drug abuse and unknown substances.

LC-MS supports high throughput analysis, making it deals for routine clinical and forensic applications.

It reduces the need for extensive sample preparation, improving efficiency and turns around time.

LC-MS techniques are widely used for quantitative analysis in bio-analytical laboratories, offering reliable & sensitive estimation of drugs and metabolite in complex biological samples.

The use of automated workflows, such as liquid -liquid extraction in 96-well plates formats can significantly increase sample throughput while minimizing manual handling.

LC-MS when combine reduces manual handling and improves efficacy**.**

The method supports accurate & reproducible measurements that are suitable for modern bioanalytic workflows [11].

The method ensures consistent, accurate results suitable for routine bioanalytical laboratories.

The method is applied for:

- Drug candidates.
- Metabolites.

It enables sensitivity & selective qualification, even at low concentration.

LC-MS supports multiple preparation techniques.

a. Solid phase extraction.
b. Liquid phase extraction.
c. Protein precipitation.

Mass spectroscopy provides high selectivity, better detection of analytes in complex mixtures.

a. Low level compounds.
b. Co-eluting substance.

LC-MS plays a key role in [12].

a. Method development strategies.
b. Variation of bioanalytical method.

5. Advantages.

LC-MS offers many advantages over traditional chromatographic techniques such as HPLC.

- **High sensitivity and precision:** LC-MS is a highly sensitive and precise analytical technique, where it can detect analytes at trace (or) lower concentration compared to HPLC-UV.
- **High selectivity & sensitivity**:LC-MS minimizes interference from biological matrices, unlike UV based methods [13].
- **Faster analysis time:** UPLC combined with MS significantly reduces run time compared to conventional HPLC.
- **Enhanced selectivity:**LC-MS reduces interference from endogenous biological compounds [14].
- Simultaneous analysis of multiple compounds capable of multiple compounds capable of analysing multiple anticancer agents & their metabolites in a single run [15].
- It requires minimal sample preparation and simpler extraction procedures.
- Enhanced detection of trace impurities & analysis.

6. LIMITATIONS

Despite its high sensitivity and specificity LC-MS is associated with several challenges such as matrix effect, ion suppression, high cost, and complex method development [16].

- (a) Matrix effect – Since the biological samples contain many components like protein, salts, lipids etc; they may interfere with the analyte during the process and effect the ionization. Therefore, proper sample preparation is required.
- (b) Ion suppression – It occurs during the process of detection since the molecules get converted to ions during detection due to presence of competent compounds analyte produces fewer ions (ion suppression).
- (c) High cost – LC-MS is of high cost when compared to other analytical techniques since it has advanced instrumentation.
- (d) Complex method development – Method development in LC-MS requires multiple parameters and these
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- Parameters should be carefully optimised if not the method will become unreliable.
- LC-MS method requires careful optimization of various parameters which make the method complex.
- Achieving both high sensitivity and selectivity simultaneously is difficult in this method.
- The ionization process in LC-MS can be affected by interfering substances, leading to variability in signal response and quantification accuracy.
- Increasing the speed of analysis in LC-MS can compromise sensitivity and resolution, creating a greater impact between getting fast output and reliable results.
- It is difficult to achieve reproducibility and reliability by using LC-MS based method.

7. STRATEGIES TO OVERCOME LIMITATIONS

- Phospholipids are the substances that cause matrix effect. One contemporary method to reduce the phospholipids-related matrix effects involves employing a combination of acetonitrile and methanol within the organic mobile phase [17-21].
- **Problem: -** Co-elution of matrix component with analyte.
  Strategy: - Modification and optimization of LC-MS and using additional techniques like ion mobility, spectrometry can be used for better resolution [22].
- **Problem: -** Change in ionization efficiency.
  Strategy: - Perform proper matrix effect assessment during method development [23].
- **Problem: -** Complete removal of matrix effect is difficult.
- **Strategy: -** Compensate using internal standards, prefer SIL-IS use IS- normalized matrix factor [24].

8. FUTURE SCOPE

- LC-MS is a highly sensitive & specific technique that is essential for pharmaceutical analysis.
- Its applications include drug metabolism studies, drug discovery analysis, identification & characterisation of impurities in drugs and its products.
- Development of chip-based, nanoflow LC systems allows higher sensitivity with smaller sample volumes, crucial for limited clinical samples.
- Increased focus on multiclass compound detection (>1000 compounds) to identify contaminants &biomarkers in complex matrices.
- Development of hybrid instruments such as; Q-TOF.
- The sample preparation techniques with multiplexed LC-MS techniques may lead to faster analysis [25].
- The latest developments in LC-MS have made 4-fold increase in the analysis without any corresponding increase in cost or loss of resolution.
- In recent years LC-MS has become a versatile tool for the quantitative & qualitative estimation of biotherapeutics.
- In recent years, LC-MS has emerged as a versatile tool for both the quantitative and qualitative analysis of biotherapeutics. Over the next decade, LC-HRMS is expected to remain the preferred method for metabolite profiling, offering a continued competitive advantage in the field of bioanalysis.

9. Applications [1].

LC-MS has wide range of applications in various fields like;

a. Life sciences.
b. Forensics.
c. Drug abuse.
d. Blood, Oral & Urine sample analysis.
e. Environmental sciences.
f. Analysis of Food Additives & Beverages.
g. Biopharmaceuticals.
h. Pharmaceuticals.

Figure 02: Applications of LC-MS in various fields [1].

Lifesciences [18]

- LC-MS has transformed life sciences by providing highly sensitive, selective & high-throughput analytical capabilities.
- It has become an essential tool in biological research that enables a deeper understanding of biochemical pathways, mechanism of actions of diseases & biomolecular interactions.

Forensics

- LC-MS has emerged as aninnovative & modern in forensic investigation.
- LC-MS play a critical role in investigation studies;by identifying the biological markers & detection of the hazardoussubstances & illegal drugs.
- LC-MS is the essential tool for forensic scientists who try to challenge the criminal situations because of its accuracy, sensitivity& adaptability [1].
- It provides a scientific framework for evaluating the evidence.
- LC-MS is increasingly used in determination of drug concentrations in biological samples obtained from pharmacokinetic or toxicological studies [19].
- LC-MS in forensic investigations isbeyond the identification of substances; it also helps todetermine the purity & concentration of the substances.

Drug abuse

Drug analysis is the primaries uses for LC-MS in forensics. Frequently, LC-MS plays a vital role in pharmacokinetic & pharmacodynamic studies.

LC-MS method has also developed for the direct analysis of 17drugs in oral fluids.

LC-MS method was validated to quantification of 30 illicit synthetic cathinones, including N-tethylpentylone&eutylone in postmortem blood [1, 20].

LC-MS is a sensitive and cost-effective tool that is alternative to ELISA for screening of urine specimens.

Blood, Oral & Urine sample analysis:

- LC-MS is crucial for evaluating blood & urine samples in forensic & therapeutic contexts & its usefulness in drug detection.
- To recognize metabolites & poisons in body fluids forensic pathologists & toxicologists depend on capacity of LC-MS.
- Blood & urine samples are used for detection & quantification of various metabolites.

Environmental sciences

- Persistent organic pollutants (POPs) concentrations in environmental samples are precisely measured by LC-MS, which help in assessing the potential risk that these pollutants pose to ecosystems & human population.
- The crucial components of ethical resource management are environmental evaluation & monitoring.
- It will detect contaminants & pollutants, even at low levels, from organic molecules to heavy metals.
- LC-MS has transformed environmental sciences by enabling the pollutant detection, analysis & management in the environmental matrices.

Analysis of Food, Additives & Beverages [1]

- LC-MS has emerged as a powerful analytical tool for detecting, identifying, & quantifying food additives, preservatives, sweeteners & veterinary drugs residues in various food matrices.
- LC-MS plays animportant role in detecting food adulteration, enhancing consumer trust & product integrity.
- LC-MS method was developed to estimate the caffeine levels in soft beverages.
- Recently, LC-MS analysis was used for the chemical characterisation of virgin & recycled polyethylene terephthalate films in food & beverages.

Biopharmaceuticals

- LC-MSis an essential, high-sensitivity tool in biopharmaceutical development for characterising complex molecules like monoclonal antibodies & vaccines.
- It helps in intact mass verification, subunit analysis, peptide mapping, post-translational modifications (PTM) identification, glycan profiling, impurity monitoring, & structural analysis.

Pharmaceuticals

- The pharmaceutical sector significantly relies on LC-MS for drug discovery, analysis & quality control to assure the safety & effectiveness of medicines.
- LC-MS provides sensitive & precise methods for measuring phospholipids which helps to formulate lipid-based drugs &understanding how these lipids influence drug delivery.
- Chiral LC-MS plays a significant role in the research & development of pharmaceuticals.
- Determining drug levels in patient samples during both preclinical & clinical trials in an essential application of LC-MS.
- A specific LC-MS method was developed & validated to quantify 5-Amino-1-methyl quinolinium in rat plasma & urine samples to evaluate its PKa& oral bioavailability.

9. REGULATORY CONSIDERATIONS [1, 16, 21]

- In developing and validating analytical methods, regulatory expectations are important for LC-MS because it demonstrates compliance with necessary standards of accuracy, precision, and reproducibility.
- Compliance with regulations from the ICH (International Council for Harmonization), USFDA and EMA (European
- Medicines Agency) is important in establishing the validity and acceptability of analytical results in pharmaceutical and clinical applications.

ICH Q2 Guidelines: These guidelines explain about how to validate analytical methods like LC-MS, HPLC etc.

FDA: FDA provides certain guidelines to ensure that the bioanalytical methods used in drug analysis are sensitive, reliable, selective, and stable.

EMA: It plays a major role in cross-checking\cross validation of the results and it checks weather proper documentation is done for all steps or not.

10. CONCLUSION

Liquid chromatography-Mass spectroscopy based bioanalytical methods have become indispensable tools for the quantitative estimation of drugs in complex biological samples. Their high sensitivity, selectivity, & accuracy make them ideal for pharmacokinetic, toxicological & clinical studies. Despite challenges such as matrix effects & method optimization, continuous advancements in instrumentation & sample preparation have significantly improved reliability & throughput. Overall, LC-MS remains a cornerstone in modern bioanalysis, with expanding applications in drug development & personalized medicine.

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13. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

14. INFORM CONSENT AND ETHICAL CNSIDERATIONS

Not applicable

15. AUTHOR CONTRIBUTIONS

Both are contributed equally.

15. REFERENCES.

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