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REVIEW

A Brief Assessment of the Bioanalytical Methods by using LC-MS/MS for the Quantitation of Melatonin: A Potential Biomarker for Sleep-Related Disorders

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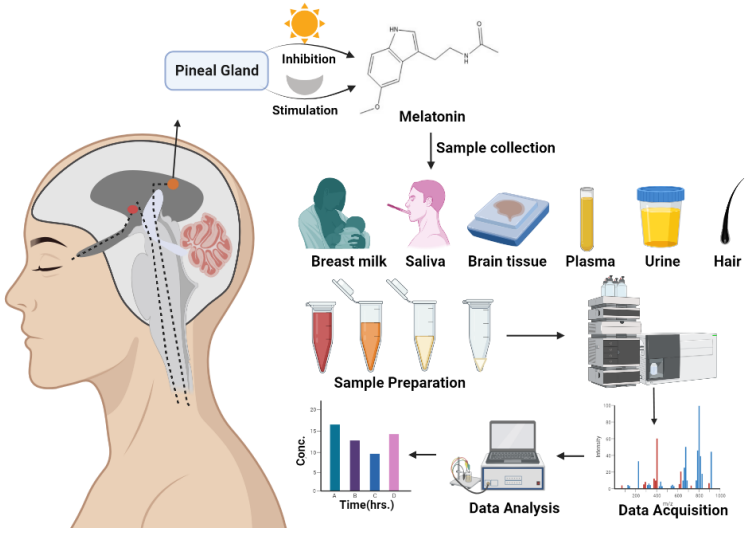

Due to its better sensitivity and selectivity, liquid chromatography with tandem mass spectrometry is the preferred choice for the quantification and identification of biomarkers, parent molecules/ metabolites in human saliva, plasma and urine etc. All such quantification methods for melatonin in biological matrices have been summarized. Melatonin is considered as a potential biomarker for circadian rhythm disturbance related disorders such as cancer, depression, insomnia, etc. Accurate quantification of melatonin is very challenging and critically depends upon the reproducibility and ruggedness of the analytical method. LC-MS/MS technique is considered as the preferred method of analysis for melatonin as compared to immunoassays. Most of bioanalytical melatonin quantification methods consist of, extraction from the biological matrix analyzing by LC-MS/MS. Our review shows that LC-MS/MS is a rugged and dependable instrument for the robust and precise quantitation of melatonin. This review compiles key elements like extraction procedure, linearity range, and chromatographic conditions.

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Keywords: Melatonin, LC-MS/MS, bioanalysis, biological matrix/matrices, endogenous molecule

List of Abbreviations

AA	Ammonium acetate
ACN	Acetonitrile
AF	Ammonium Formate
API	Atmospheric Pressure Ionization
CAS No.	Chemical Abstracts Service Number
CC	Calibration Curve
ESI	Electrospray Ionization
FA	Formic Acid
IPA	Isopropyl Alcohol
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid Chromatography
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LLE	Liquid–liquid extraction
Log P	Partition coefficient
mM	milli-Molar
MPA	Mobile Phase A
MPB	Mobile Phase B
ng mol^{-1}	Nano gram per mole
pH	logarithm of the reciprocal of the hydrogen ion activity
pKa	Dissociation Constant
PK	Pharmacokinetic
pg mol^{-1}	Pico gram per mole
PP	Protein precipitation
SPE	Solid-phase extraction
SPME	Solid phase micro extraction
UPLC	Ultra-Performance Liquid Chromatography

INTRODUCTION

Melatonin is a naturally occuring indole amine, which is chemically identified as N-acetyl-5-methoxytryptamine (Figure 1). The human body has many sources for the production of melatonin. Its physicochemical and pharmacokinetic properties are listed in Table I. In humans, the pineal gland is the major source of melatonin secretion however ocular light exposure hinders this. This secretion and inhibition process is controlled by the hypothalamic suprachiasmatic nucleus hamster clock.[1] The suprachiasmatic nucleus of the hypothalamus is the biological clock that modulates melatonin production and secretion over the full day span of 24 h.[2]

Melatonin levels are generally increased during the night hours, but these levels start dropping with the progression of the morning and decrease further throughout the day. Raised night levels of melatonin facilitate target organs to enter into an appropriate homeostatic metabolic rhythm helping the human body to protect itself from developing various diseases.[3] Therefore, subjecting the body to light at night may result in a disturbance in the production and secretion of melatonin hence disrupting the circadian rhythm. The human body has specific receptors for melatonin through which it regulates various physiological functions.[4] Apart from the pineal gland, other organs that synthesize melatonin are gastrointestinal-tract, lymphocytes, bone marrow and, skin.[5]

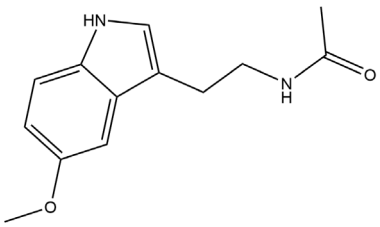

Figure 1. Melatonin chemical structure.

Plants have a different synthesis pathway of melatonin than animals. In plants, decarboxylation of tryptophan happens first, followed by hydroxylation resulting in the formation of serotonin. In animals, hydroxylation of tryptophan happens first followed by decarboxylation leading to serotonin formation. Serotonin is further acetylated and methylated to form melatonin.[6]

To start the production of melatonin, tryptophan serves as a starting molecule in cell species, followed by other steps like decarboxylation, methylation, hydroxylation, acetylation, etc. However, the order in which these steps take place varies from species to species. Serotonin first undergoes acetylation followed by methylation to form melatonin. The synthesis of melatonin is represented in Figure 2.

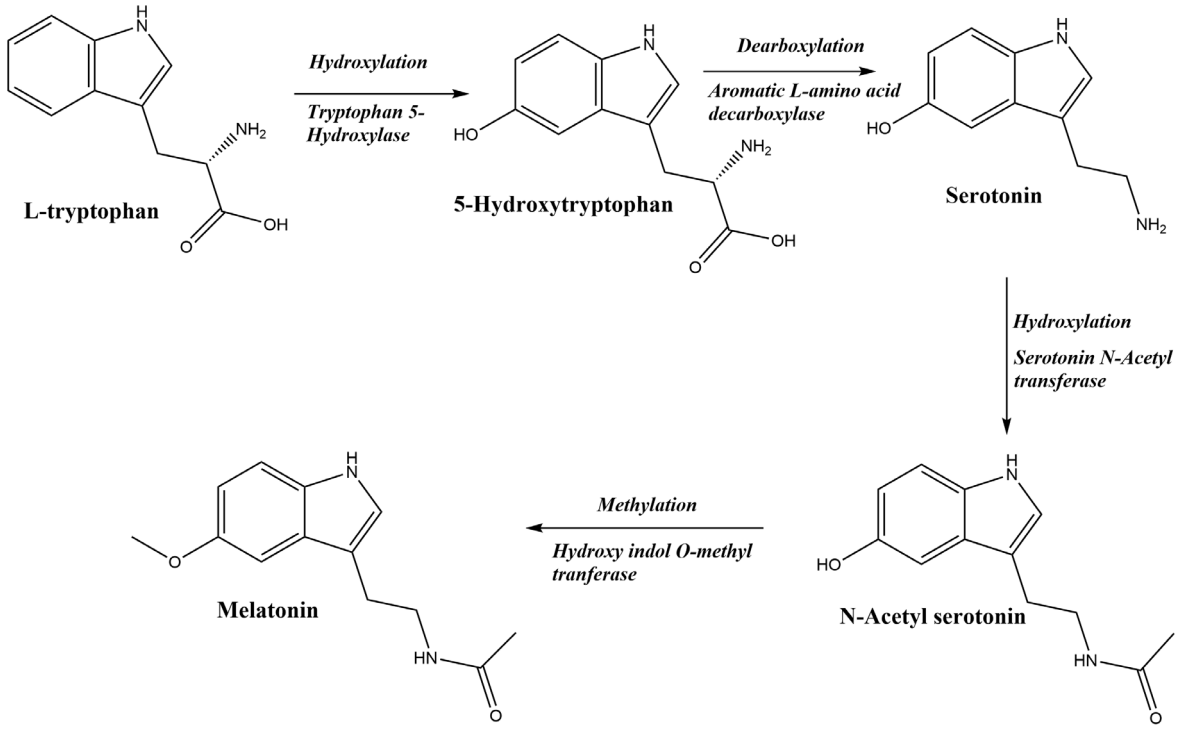

Figure 2. Synthesis of melatonin.

Melatonin is an endogenous hormone and potent neurotransmitter that helps regulate circadian rhythm and sleep. Modulation of the body temperature, hormone levels, sleep, and metabolism is done by the biological clock (also called as inner clock) by changing the physiology as per the different durations of the day.[7] Disturbance in this inner clock may lead to grave consequences for mental as well as bodily well-being.[8]

For example, the scientific evidence advises that sleep-wake cycles affect the hormone regulation and showing misaligned behavioural models (e.g., shift workers), so, it results by governing the risk of type 2 diabetes, obesity, cancers, and coronary diseases.[9,10]

A recent study has demonstrated that youngsters with disturbance in circadian cycles are more prone to develop drug and or alcohol abuse.[11,12] Learning issues and cognitive impairments.[13] Since the circadian rhythms regulate various hormone levels including melatonin, such hormones can be used as biological markers or pharmacodynamic markers for diseases caused by a disruption in the circadian rhythms.

Melatonin is commonly used in the treatment of sleep disorders such as insomnia and jet lag, for the reestablishment of circadian rhythms in cases of blindness and shift works. It has also found application in the prevention of developing cancer, as adjuvant therapy in cancer, and to slow down neurodegenerative diseases. Melatonin is also a highly effective antioxidant and free radical scavenger. One of the most studied actions of melatonin is its antitumor effects, which include antiangiogenic effects in several types of tumors. Additionally, melatonin plays an important role in regulating glucose metabolism and has been found to have significant anti-proliferative and apoptotic effects on various cancer cells. This hormone is also known for its pharmacological action in reducing oxidative conditions, as well as its ability to reduce inflammation and modulate immune responses (Figure 3).

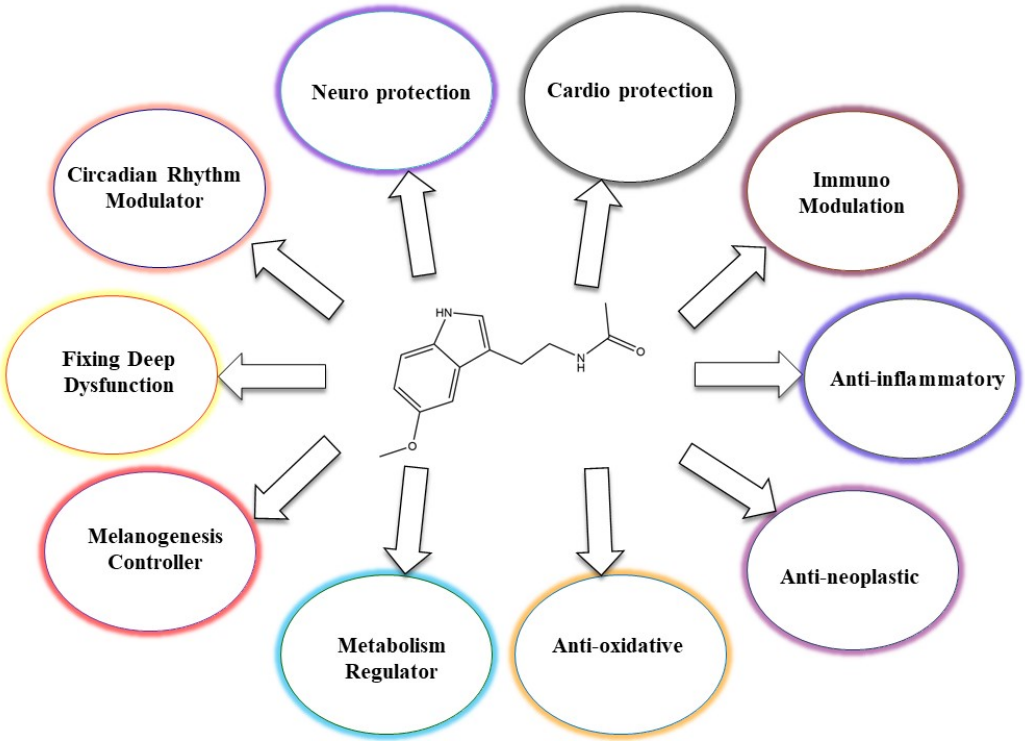

Figure 3. Schematic overview of other main functions of melatonin.

There are several sample types which can be used for the measurement of melatonin as well as testosterone, for example blood,[14-16] urine,[17,18] and saliva[19,20]. However, due to the pain free and ease of sampling, saliva is the most desired sample type.[21] Numerous published literature studies show that melatonin, testosterone, and cortisol all have well established correlations between hormone levels of serum versus saliva.[22-24]

The success of any treatment solely depends upon the drug's method of action, the patient's trust in the treatment (hence religiously following the regimen), and the optimum drug level in the blood. Bioanalytical methods are best suited to check the drug level in the biological matrices and also to evaluate patient adherence.

These bioanalytical methods have a noteworthy role to play in various therapeutic drug monitoring studies, toxicology or pharmacological evaluation of bioavailability, and PK (pharmacokinetic) evaluation.[25-27] Before subjecting the bioanalytical method to such usage, it has to be fully assured that the method is

fit for the intended analysis by appropriate validation experiments. Bioanalytical method validation and study sample analysis (ICH M10) provide recommendations for biological and chemical drug quantification and their application in the analysis of study samples. However, if any significant changes in method validation approach applicants can consult their respective regulatory authorities by providing proper justification. Various regulatory specific guidelines for bioanalytical method validation are published and updated from time to time by regulatory agencies like Food and Drug Administration and Therapeutic Goods Administration[25] European Medicines Agency.[28]

For various non-clinic and clinic usage, precise and accurate quantification of endogenous compounds is of utmost importance. For absolute quantification, the standard curve needs to be prepared in the same matrix as that of the unknown samples, to nullify the difference in extraction recoveries and the matrix effect. However, for endogenous compounds, it's not feasible to get a drug free matrix due to the endogenous nature of the drug.

LC-MS/MS analyses are a crucial aspect of bioanalytical research in pharmaceutical industry. However, one concern that arises in these analyses is the potential matrix effects, which can result in erroneous results. To address this concern, several approaches are used namely, surrogate matrix, surrogate analyte, background subtraction, and standard addition.

Surrogate matrix is prepared by stripping off the endogenous drug using some common adsorbent (like activated charcoal) to generate drug-free matrix which are then used to prepare calibration curve standards.[29-31] During charcoal stripping, this has to be ensured that all the charcoal particles are efficiently removed before spiking with analytes of interest. This may lead to significant false reduction of the quantified analyte concentration if not done efficiently.

The use of surrogate analyte approach in method development is easy when there is availability of stable-isotope-labeled standards. On the other hand, the surrogate matrix method requires extensive initial method development efforts; however, the benefit is that it simplifies sample analysis in the long term.[32]

The use of the standard addition method offers the advantage of utilizing the identical matrix of each study sample to construct its own calibration curve. Additionally, this method enables direct quantification of endogenous analytes, without requiring manual subtraction of background peak areas.

On the other hand, the surrogate matrix approach involves using different matrices, including artificial, stripped, and neat matrices, as substitutes for the actual matrix.[33] The measurement of melatonin in serum/plasma is further difficult due to 'Melatonin' being an endogenous substance with sub-nanogram levels in biological fluids, imposing serious challenges in developing and validating a highly sensitive bioanalytical method using. This issue has been addressed using water[34] and charcoal stripped plasma[35] as a surrogate matrix to prepare a standard curve.

Two major challenges are generally faced in melatonin quantification – basal melatonin levels (due to endogenous nature) and the desired sensitivity. The melatonin levels in the human body are very dynamic and are not same in day and night. So, blood/plasma collected for the preparation of standard and quality control samples have varying degree basal melatonin level which will give erroneous quantification and QC failure issues. The use of charcoal stripped matrix helps to get rid of this issue. Also, the correct basal value estimation for the clinical trial or patient samples (which are normally in sub nanogram levels) is very important to understand the requisite prescription dose for the patient. Hence, the sensitivity of the quantification method becomes further important.

In recent times, various bioanalytical techniques have been reported by overcoming the imposed challenges to improve the melatonin quantification using the powerful quantification instruments like LC-MS/MS. The main aim of this review article is to collate the various published LC-MS/MS based melatonin bioanalytical quantification methods in different biological matrices like serum, plasma, milk and saliva etc. Accordingly, this review has been performed by doing a literature search using web of science, Google scholar and PubMed. The keywords used are melatonin, biological matrix/matrices, LC-MS/MS, mass spectrometry, bioanalysis, validation, pharmacodynamics, pharmacokinetics. Physicochemical and pharmacokinetic properties of melatonin were given in Table I.

Table I. Melatonin physicochemical and pharmacokinetic properties

Physicochemical Properties	
Chemical Composition	$C_{13}H_{16}N_2O_2$
IUPAC Name	N-[2-(5-methoxy-1H-indol-3-yl)ethyl] acetamide
Physical Description	Solid
Color/Form	White-cream to yellowish crystalline powder
Melting Point	117 °C
Solubility	>34.8 [μg mL^{-1}] (The mean of the results at pH 7.4)
LogP	1.6
PKa	16.51 and -0.69 and is uncharged in the entire pH range
Mol. weight	232.28 g mol^{-1}
CAS No.	73-31-4
Pharmacokinetic Properties	
Half-life	35 to 50 minutes
Majorly metabolized	Liver
Oral bioavailability	3-15%
Major route of elimination	Urine

Types of Extraction Methods Used in Sample Preparation

Sample clean-up is another way to enhance the extraction efficiency of any biological method resulting in better sensitivity and specificity. Various samples like liquid-liquid extraction, protein precipitation and solid phase extraction are the commonly employed ones for the sample cleanup prior to LC-MS analysis.[36]

Solid-phase extraction (SPE)

SPE is an extractive technique employing a solid bed in the form of a cartridge which acts as an extraction or separation media when liquid and/or treated matrix samples containing desired analytes are passed through it under the influence of positive or negative pressure. It is thus far the most effective and reliable extraction procedure especially for the separation of analytes of interest from the complex matrices like blood, tissues, plasma or urine. This technique not only separates the unwanted interferences from the desired analyte but a very effective way of concentrating the analyte also resulting in the enhanced method sensitivity especially required for the complex molecules like melatonin. Out of the 21 methods reported in the compilation Table II, 7 methods have employed solid phase extraction for melatonin sample clean-up.

Liquid–liquid extraction (LLE)

LLE is an extraction technique where the analyte gets partitioned between the two immiscible solvents based on its affinity resulting in its separation. This extraction technique is generally used in case of less complex matrices like saliva. Out of the 21 methods reported in the compilation Table II, 8 methods have employed LLE for melatonin sample clean-up. The biggest disadvantages of LLE include the use of large solvent volumes. Also, the organic solvents generally used for LLE are highly carcinogenic, resulting in a health hazard for the user.

Protein precipitation (PP)

Protein precipitation is the desired method of extraction, especially for the discovery samples where least method developments are desired. Out of the 21 methods reported in the compilation Table II, 4 methods employed protein precipitation for melatonin sample clean-up. PP is a simple, fast and cost-effective extraction method as the precipitation is done by using commonly available laboratory reagents like acetonitrile or trifluoroacetic acid, or ammonium sulfate. PP has relatively poor sample cleanup and therefore, Due to the presence of phospholipids and oligosaccharides that are not completely removed, it is connected with high levels of ion enhancement or ion suppression. The only way to resolve high levels of ion enhancement or ion suppression is to use a deuterated IS that co-elutes with its respective analyte. As a result, protein precipitation was deemed the best method for estimating 19 analytes simultaneously in breast milk using LC-MS/MS.[37]

Solid phase micro extraction (SPME)

SPME is a modern and highly sensitive method of sample preparation that does not require a solvent. This innovative technique operates on the principles of adsorption and absorption, followed by desorption. Out of the 21-method reported in the compilation Table II, one method has been employed SPME for melatonin sample clean-up.

Obtaining precise, sensitive, accurate and results in analysis of samples can be challenging due to the complexity of sample matrices. To mitigate sample interference effects from the environment and enhance the detection of the main analyte, prior to analysis it is crucial step to integrate suitable sample preparation method. There are some alternative and more advanced sample preparation method discovered to achieve the maximum efficiency of the main analyte of interest. Presently, there are multiple sample preparation techniques in use including, liquid-phase micro-extraction, magnetic solid-phase extraction, dispersive solid-phase extraction and stir-bar sorptive extraction and QuEChERS (quick, easy, cheap, effective, rugged, and safe), Microextraction in packed sorben, Stir-Bar sorbent extraction and Fabric phase sorptive extraction. These advanced extraction methods were used in the detection of samples of breast milk and cow milk using LC-MS/MS techniques. But no such method has been reported with respect to melatonin determination with LC-MS/MS.[38]

Till date, no such systematic review has been done for the measurement methods of melatonin in biological matrices using LC-MS/MS. Table II represents published assays covering main aspects of internal standard, sample extraction, mobile phase, CC range, and instrument used for the LC-MS/MS quantification of the melatonin in complex biological matrices.[38] The prepared pie chart indicates contribution of the use of each sample preparation techniques (Figure 4) as per the available literature.

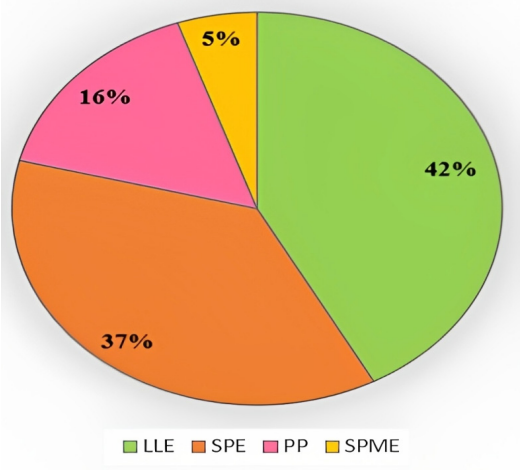

Figure 4. Pie chart representation of mostly used methods for sample preparation (LLE: Liquid-Liquid Extraction, SPE: Solid-phase extraction, PP: Protein precipitation, SPME: Solid phase micro extraction).

Table II. Compilation of the published assays for the quantitation of melatonin

S. No	Matrix	Internal standard	Sample Extraction	Mobile Phase	Instrument	Chromatographic conditions	Calibration Curve Range	References
1	Human Plasma	Melatonin-D4 6-OH-Melatonin-D4	PP and SPE	MPA: 7.5 mM AF in H_2O pH 2.65 MPB: 0.1% FA in ACN	Acquity UPLC coupled with Xevo TQ-S with electrospray ionization source.	Column: Acquity BEH C18, 130 Å, 1.7 µm, 2.1 × 100 mm column (Waters) Column temp.: 35 ºC Autosampler temp.: 10 ºC Injection Volume: 10 µL	Lowest concentration 2.2 nM for serotonin to 1.0 pM for 6-OH-Melatonin	35
2	Human Saliva	Not available	LLE	NA	shimadzu Nexera X2 UPLC coupled with Sciex QT6500 mass spectrometer	–	0.78 to 100 pg mL^{-1}	39
3	Breast Milk	Not used	PP	Mode: Isocratic 0.1% FA in ACN and 0.1% FA in H_2O (Ratio not found)	4000 QTRAP from Sciex	Column: Kinetex C8 column (30 × 3 mm, 2.6 µm) Column temp.: 45 °C Autosampler temp.: 5 °C Injection Volume: 4 µL	10 to 500 ng mL^{-1}	40
4	Human Milk	Melatonin-D4	PP using ACN	MPA: 2 mM AF in H_2O MPB: 0.1% FA in ACN	Waters 2D UPLC equipped with TQ-XS triple quadrupole mass spectrometer	Column: ACQUITY UPLC HSS T3 column (50×2.1 mm, 1.8 µm) Column temp.: 45 °C Injection Volume: 30 µL	1 to 1000 pg mL^{-1}	41
5	Human Urine	Melatonin-D4	SPE	MPA: 0.1% FA in H_2O MPB: 0.1% FA in ACN	Agilent 1290 LC coupled to a 6500 QTrap equipped with an electro spray ionization source	Column: Kinetex® C18 column (50 × 2.1 mm, 2.6 µm) from Phenomenex Injection Volume: 20 µL Flow rate: 600 µL/min	Melatonin: 7.5 to 500 pg mL^{-1} 6 Hydroxy melatonin: 375 to 25000 pg mL^{-1}	37

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Table II. Compilation of the published assays for the quantitation of melatonin (continuation)

S. No	Matrix	Internal standard	Sample Extraction	Mobile Phase	Instrument	Chromatographic conditions	Calibration Curve Range	References
6	Human Salivary Samples	Melatonin-D4 Cortisol-D4	LLE	MPA: 2 mM AA in H_2O MPB: 0.1% FA in ACN	Agilent 6490 ESI source in positive mode.	Column: C18 2.1×50 mm 2.6 µm Kinetex Injection Volume: 20 µL Flow rate: 250 µL/min Run time: 6 min	Melatonin: 2.15–430 $pmolL^{-1}$ Cortisol:0.14–27.59 $nmolL^{-1}$	42
7	Rat Serum Samples	D4-Melatonin D4 -Cortisol L-Thyroxine-13C6	LLE	MPA: 0.1% FA in H_2O MPB: 0.1% FA in CH_3OH	Agilent 6490 LC-MS/MS with electrospray ionization source	Column: Eclipse Plus C18 column 4.6 mm × 100 mm, 3.5 µm particle Agilent ZORBAX Autosampler- 4 ºC Injection Volume: 15 µL Flow rate: 1 mL/min	Melatonin: 0.004-0.5 $ng\ mL^{-1}$ Cortisol, T3, T4 and Testosterone: 0.4-50 $ng\ mL^{-1}$	43
8	Saliva Plasma	Cortisol-D4 Melatonin-D4	SPE	NA	Xevo TQ-MS triple in positive ionization mode.	Column: Phenomenex® Luna Phenyl-Hexyl 2.0×100 mm, 3 µm Run time: 6.5 min	In saliva Melatonin: 15–2000 $pmolL^{-1}$ Cortisol: 0.60–75 $nmolL^{-1}$. In plasma: Melatonin:15–2000 $pmolL^{-1}$ Cortisol: 40–5500 $nmolL^{-1}$	44
9	Human Saliva	Melatonin-D4	SPME	50% H_2O and 50% ACN	API 4000 from AB Sciex, in positive ion mode	Column: Inertsil ODS-3 column (50 mm × 2.1 mm, 5 µm) Column temp.: 30 °C Flow rate:0.2 mL/min	0.2 – 50 $pg\ mL^{-1}$	45
10	Dog Plasma	Desvenlafaxine	LLE with ethyl acetate	40% of CH_3OH: 60% of 5 mM AA: 0.1% FA	AB Sciex	Column: C18 Luna (2.0 × 50 mm, 3 µm) Column temp.: 35 °C Autosampler: 4 °C Injection volume: 10 µL Flow rate: 0.20 mL/min	0.020–10 $ng\ mL^{-1}$	46

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Table II. Compilation of the published assays for the quantitation of melatonin (continuation)

S. No	Matrix	Internal standard	Sample Extraction	Mobile Phase	Instrument	Chromatographic conditions	Calibration Curve Range	References
11	Human Milk Samples	7-D Melatonin	SPE	MPA: 80% of 5 mM AF in H_2O and 20% CH_3OH MPB: CH_3OH	Agilent 6460 with ESI source	–	1 to 150 pg mL^{-1}	47
12	Oral Fluid Samples	Melatonin-D7 Cortisone-D7 Cortisol-D4	Cotton swab with a rotator, Samples were extracted and purified, separated by using a Turbo Flow	MPA: Water, ACN and (mixture-1 containing 45% of ACN ,45% of IPA and 10% dimethyl ether) and mixture-2 consists of 0.1% of ammonium hydroxide in ACN) MPB: 1.5 mM AF: 30 mM FA in $CH_3OH$and H_2O	LC-MS/MS with ESI source.	Hypersil Gold column 50 mm × 2.1 mm, 3 µm	Melatonin :0.004 to 0.431 Cortisone: 5.5 to 277.4 Cortisol: 0.55 to 27.59 $nmolL^{-1}$	48
13	Salivary Samples	Melatonin-D7	To a diluted saliva sample, internal standard was added into the auto sampler vial and injected directly.	MPA: 0.1% FA in H_2O MPB: 15% CH_3OH in ACN with 0.1% FA	API-3200 tandem mass spectrometer Analyst software.	Column: Symmetry C8 (150×3.9 mm internal diameter; 5 µm) Injection Volume: 50 µL Flow rate: 0.8 mL/min Run time: 8 min	3.91- 1000 pg mL^{-1}	49
14	Sea Lamprey Brain Tissues	Melatonin-D7	SPE	MPA: 1 mM of perfluorohexanoic acid in water MPB: ACN	Shimadzu LC with Quattro micro mass spectrometer from waters.	–	0.5 -100 ng mL^{-1}	27

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Table II. Compilation of the published assays for the quantitation of melatonin (continuation)

S. No	Matrix	Internal standard	Sample Extraction	Mobile Phase	Instrument	Chromatographic conditions	Calibration Curve Range	References
15	Human Plasma	N-acetyl tryptamine	LLE with dichloromethane	MPA: 2 mM AF in H_2O with 0.1% FA MPB: ACN	API 6460 LC-MS/MS in positive electro spray ionization mode.	Column: ZORBAX Eclipse XDB C18 (4.6 mm× 150 mm, 5 µm) Column temp.: 20 °C Injection Volume: 10 µL Flow rate: 0.5 mL/min Runtime:11 min	1–5000 pg mL^{-1}	34
16	Human Plasma	Melatonin-D4	LLE	MPA: 95% of 0.2% acetic acid in $H_2O$and 5% of ACN MPB: 5% of H_2O and 95% of ACN	TSQ coupled with an Ion Max HESI source.	Waters Symmetry Shield RP18 column (2.1×100 mm, 3.5 µm) Column temp.: 50 °C Autosampler temp.: 5 °C Injection volume: 20 µL	Melatonin: 1165–116500 pg mL^{-1} N-acetyl serotonin: 10.95 – 1095 pg mL^{-1}	50
17	Human Saliva	Melatonin-D4 Cortisol-D4 Testosterone-D3	LLE	MPA: 2 mM AA with 0.1% FA in H_2O MPB: 2 mM AA and 0.1% FA in CH_3OH	Agilent 6460 operated in positive ion mode with Electrospray ion source	Column: Kinetex C18 2.1 mm × 50 mm 2.6 µm Column temp.: 40 °C Autosampler temp.: 8 °C Flow rate: 0.5 mL/min	Melatonin: 15.0–579.4 $pmolL^{-1}$ Cortisol: 0.5–90 $nmolL^{-1}$ Testosterone: 15.6–622.8 $pmolL^{-1}$	51
18	Human Saliva	Melatonin-D7	SPE	MPA: 20 mM AA in 90% H_2O and 10% CH_3OH MPB: 5 mM AA in 50% H_2O and 50% CH_3OH	Finnigan AQA mass with an electrospray ionization source	Column: silica-based octadecyl silica (ODS) column Capcell Pak C18 AQ, dp 5 µm, 2× 150 mm; Shiseido Column temp.: 40 °C	5–1000 pg mL^{-1}	52
19	Human Saliva	7-D-Melatonin	SPE	40 to 95% CH_3OH for 3 min and 95% CH_3OH for 2 min	API 2000 with an APCI source	Waters Symmetry C8 150 mm× 3.9 mm	3–300 pg mL^{-1}	19

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Table II. Compilation of the published assays for the quantitation of melatonin (continuation)

S. No	Matrix	Internal standard	Sample Extraction	Mobile Phase	Instrument	Chromatographic conditions	Calibration Curve Range	References
20	Human Serum	N-acetyl tryptamine	On-Line Sample Extraction	95% of ACN and 5% of 5 mM AF pH 4	Triple quadrupole mass spectrometer with HP1100 variable wavelength UV detector	Oasis® HLB extraction cartridge Waters column (2.1×20 mm) Flow rate: 0.2 mL/min Run time: 9 min	0.500 to 200 ng mL^{-1}	53
21	Human Plasma	Melatonin-D7	LLE	17.5% of ACN in 25 mM FA in H_2O	LC/MS system HP 1100 mass spectrometer with electrospray interface.	–	Melatonin: 4 to 80 ng mL^{-1} Hydroxy melatonin 20 to 400 ng mL^{-1}	54
22	Human Hair	d4-melatonin, d3-N-acetyl-serotonin, d4-6-hydroxymelatonin, d4-6-sulfatoxymelatonin	SPE	MeoH: 5 mM ammonium acetate (95:5% V/V)	ABI 3200 QTRAP tandem mass spectrometer-ESI positive ionization mode	Column: ODS Plastisil C18 (5 µm, 150 mm × 4.6 mm; Dikma) Column temp.: 25 °C Volume: 10 µL Flow rate: 200 µL	0.1 pg/mg to 1.0 pg/mg	55

Method Validation Parameters

In the literature various studies were analysed for the compilation of the bioanalytical method validation parameters depicting that the method developed and used were reliable and reproducible for intended use.

Selectivity is the ability of an analytical method to differentiate and measure the analyte, interfering substances and internal standard. Magliocco et al. depicted the method to be very selective as no interfering peaks were observed at the retention time of the compound in water and urine matrix which were collected from different volunteers in late night hours,[37] Zhao et al., reported the non-observance of interfering peaks at the RT of melatonin and used Internal standard in the tested blank matrix.[39]

Carry-over is the appearance of the analyte from previous injection in the current chromatogram. Karunanithi et al., and Fustinoni et al., reported carryover problem was not observed.[40,41]

Specificity is the capability of the developed method to differentiate the analyte from its related substances. Karunanithi et al., reported specificity by analysing 20 blank milk samples. The used blank milk batches were first treated with activated charcoal for overnight resulting in the absorption of the matrix melatonin hence making them free from melatonin The melatonin free milk samples (n =20) were analysed for specificity.[40]

Extraction Efficiency/Extraction Recovery is the amount of the analyte recovered after sample clean up processing steps of the extraction method. The percent efficiency/recovery of analyte was calculated with respect to response of the same amount of standalone standard analyte. Demeuse J. et al., reported recovery or the extraction efficiency above 70%. Monfort et al., reported the extraction recovery ranging from 87.3 to 110.8% with % CV ranging from 2.3 to 14.8%.[42,43]

The matrix effect is generally evaluated to monitor the effect of different matrix or sources. Monfort et al., reported the Matrix effects variance RSD of 11.4%.[43] Magliocco et al., reported the mean IS-normalized matrix effects between 100–109% (RSD ≤ 5%) indicating absence of any significant matrix effect.[37]

Method Precision and Accuracy

Weiqi Jin et al., depicted inter-day and intraday batch accuracy and precision on three validation batches. Each batch consisted of six replicates of Quality control samples in human breast milk samples at higher, mid and low levels. The intra and inter-assay precision depicted 4.1% and 6.8% RSD respectively.[44] Magliocco et al., reported the accuracy data within the acceptance criteria range for melatonin and 6-hydroxymelatonin 92.4–104.6% and 94.0–102.6%, respectively (85–115% of the theoretical value). Precision values for all QC samples of both analytes were within 15%. Good repeatability was depicted i.e., 3.4–10.4% for melatonin and 4.2–7.9% for 6-hydroxymelatonin.[37]

Reinjection reproducibility, as the name suggests is assessed by reinjecting a run at low, middle and high QCs after storage for appropriate duration and temperature conditions. The precision and accuracy of the reinjected QCs decides the validity of the processed samples. Weiqi Jin et al., reported re-injection of processed-sample after 48 h and compared with first analysis i.e., 0 h. The accuracy was depicted to be within ±12.3% deviation, and the precision was depicted to be 2.8% RSD.[44]

Dilution integrity depicts the method ruggedness in case the matrix sample needs to be diluted, if the sample volume is not sufficient or it's required to be diluted to bring within the calibration curve range Monfort et al., reported dilution integrity, to affirm the sample integrity if samples are to be diluted, at dilution factors of 10x and 100x. The accuracy was depicted to be within (85.0–113.5%) after dilution and precision was shown to be (1.4 –10.1%).[43]

Stability of the analyte in the matrix is evaluated using low and high concentration quality control samples. Aliquots of the low- and high-quality (and sometimes mid also) control samples are analysed at time zero (freshly) and after the stipulated storage requirement. The mean concentration at each quality control samples level should be within ±15% of the nominal concentration.

Monfort et al., reported short-term stability at room temperature for one day and at -20 °C for 14 days. Long term stability was evaluated by analysing lower quality control samples and high-quality control

samples after storing them at -80 °C for 30 days. Freeze-thaw stability was evaluated for 3 cycles of freezing at -80 °C and unassisted thawing at room temperature and all the results were found to be within the acceptance criteria.[43] Zhao et al., depicted the melatonin stability in dog plasma. The short-term plasma stability at room temperature was depicted to be 4 hr and long-term plasma stability at -70 °C was depicted to be for 25 days. Also, the sample were found to be acceptably stable after three freeze thaw cycles and in processed samples at 4 °C for 24.[39]

DISCUSSION

Some distinctive procedures are covered in this section to demonstrate variations in the bioanalysis of melatonin. Firstly, quantifying melatonin in various biological matrices that is, endogenous substances with ultra-low levels make it difficult. The blank of the biological matrix is not "blank". This leads to a serious issue in the validating a method by LC-MS/MS which appeared to be difficult to removal by using the regular procedures for overcoming the same water used as calibration matrix An-Qi Wang et al. (2011)[34] and in few cases, charcoal pretreatment Duraisamy Karunanithi et al. (2013)[47] was carried out to eliminate interference.

Sebastian Hartter et al. (2001) reported a bioanalytical method for estimating melatonin and its major metabolite in plasma, including enzymatic hydrolysis and one-step liquid–liquid extraction using 17.5% ACN in FA in H_2O and separation was done by using Luna C-18 column. As reported in Table II, the method was used for the plasma sample analysis from the healthy volunteers after administering a single 35 mg dose via the oral route. The C_{max} for melatonin & 6-hydroxymelatonin was observed to be 19.2 ng mL^{-1} & 694 ng mL^{-1} respectively. With a T_{max} of 1.5 hours for both analytes after melatonin intake.[54]

Shuming Yang et al. (2002) reported a fast measuring of melatonin in human serum by LC-MS/MS. N-acetyl tryptamine was used as ISTD. It applies ESI–MS–MS detection along with online preparation of the samples. Using this method, unextracted serum samples were injected directly. The technique has a wide linear range, is precise and sensitive and has excellent reproducibility with a total run time of 9 minutes per injection. This method can be used for the pharmacokinetic study of melatonin in human serum and blood.[53]

The estimation of melatonin was reported by Kare Eriksson et al. (2003) using HPLC–MS/MS. The limit of quantification and limit of detection was 3.0 pg mL^{-1} and 1.05 pg mL^{-1} respectively. This reported method depicted better sensitivity and increased specificity for melatonin quantification in saliva samples and the results were observed to be more reliable than immunoassay.[19]

Motoyama et al. (2004) reported a bioanalytical method for direct estimation of endogenous melatonin using Mobile Phase A (MPA) as 20 mM of AA in 90% H_2O and 10% CH_3OH and Mobile Phase B (MPB) as 5 mM of AA in 50% H_2O and 50% CH_3OH using ODS C18 column. The developed method by Motoyama et al. was the very first method to perform direct quantification of endogenous levels of melatonin is human saliva samples using the column switching technique. The method depicts a good throughput due to its smaller run time resulting from the minimal sample pretreatment. The method has been validated for 5–1000 pg mL^{-1} and can be very useful for melatonin clinical trials.[52]

The first method for estimation of melatonin and cortisone in saliva in a single run was reported by Jensen MA et al. (2011) Samples were extracted using liquid-liquid extraction.[51]

Melissa D. et al. (2011) reported a method by using nano flow LC-MS/MS and electro spray LC-MS/MS for estimation of melatonin and nor melatonin in human plasma by Gradient Elution using 95% of 0.2% CH_3COOH in H_2O and 5% of ACN (95:5) in MPA and 5% of H_2O and 95% of acetonitrile (5:95) in MPB. Waters symmetry shield RP 18 column was used for separation of human plasma by using liquid–liquid extraction. This method used nano-LC for sample injection and was validated over range of 11.65–1165 pg mL^{-1} & 10.95–1095 pg mL^{-1} for melatonin and N-acetyl serotonin respectively. The usage of nano-LC has resulted into enhanced sensitivity for endogenous melatonin levels up to sub-picogram levels.[50]

An-Qi Wang et al. (2011) validated bioanalytical method in plasma for melatonin by LC–MS/MS using water as a calibration matrix by using 2mM ammonium formate and 0.1% FA in H_2O in MPA and ACN

in MPB and chromatographic separation was carried out on XDB C18 column manufactured by Agilent. Two complementary quality control approach were used to validate the method. The PA batches for low concentration (1 pg mL^{-1} and 10 pg mL^{-1}) and potential matrix effect experiments were run using water as matrix whereas pooled plasma was used to perform PA batches and matrix effect at high concentration (50, 500 and 5000 pg mL^{-1}). The accomplished method was employed to plasma melatonin exogenous and endogenous levels quantification for dog samples.

The developed method was employed to check the PK profile of exogenous melatonin and daytime baseline level of endogenous plasma melatonin in beagle dogs after oral administration.[34]

Huiyong Wang et al. (2012) first reported an estimation of melatonin and neurotransmitters in brain tissues by LC-MS/MS. Separation was done by using a reversed-phase column with MPA as 1 mM of perfluorohexanoic acid in water and MPB as ACN. SPE Extraction method was used to extract and purify the analyte from the tissue of brain samples. Different types of solid phase extraction beds were tried during method development resulting in the extraction recoveries from 71.3 – 95.3%, however the best recovery was obtained using Bond-Elut C18 cartridges. The limit of detection for both the analytes were less than 200 pg mL^{-1}. The method described here is appropriate for quantifying norepinephrine, dopamine, 5-hydroxytryptamine and melatonin levels in biological samples with high accuracy, reproducibility and low inter and intra-day variation.[56]

Sohil A. Khan (2013) reported a LC-MS/MS technique for observing sleep disorders in children with melatonin concentrations in saliva. The separation was done by using a gradient mobile phase 0.1% FA in H_2O in MPA and 15% CH_3OH in ACN with 0.1% FA in MPB with C8 column. The method was found to be depicting no matrix effects and was successfully applied clinically for both children and adult samples for diagnosis purpose.[49]

First automated assay for the parallel estimation of melatonin, cortisone and cortisol in oral fluid samples was reported by Fustinoni S. et al. (2013) by LC/MS in saliva by using turbo flow system was operated by using 2 sets of mobile phases. The MPA consists of H_2O, ACN, mixture-1 (containing 45% of ACN, 45% of IPA and 10% DME) and mixture-2 (consists of 0.1% of NH_4OH in ACN). The MPB consists of 1.5 mM AF to 30 mM FA in CH_3OH and H_2O.[48]

This assay qualifies for all the checks of an ideal method like high-throughput, requirement of small specimen volumes with very minimal manual interventions. These characteristics along with the non-invasive sample collection (oral fluid) has led to its application in epidemiological studies.

Quantification of melatonin in milk was validated by using LC-MS/MS and reported by Duraisamy Karunanithi et al. (2013) in which solid phase extraction cartridges were used to purify dichloromethane before it was used to extract the samples.[47]

Earlier developed methods for melatonin quantification in milk samples using radioimmunoassay have severe selectivity concerns due to the cross reactivity from the milk components. Fluorescence detection with HPLC and GC-MS were found to be having good sensitivity but the method was time consuming due to the involvement of derivatization step.

This LC-MS/MS method was reported to be more selective and specific. Further a better LOD without the tedious derivatization can be obtained. This LC-MS method was employed for melatonin quantification of milk samples.

Huimin Zhao et al. (2015) developed a method for melatonin measurement in dog plasma, and the dog plasma samples were prepared using ethyl acetate as solvent by liquid-liquid extraction method and separation was done by using C18 column with isocratic mobile phase. The reported method has been employed to measure the melatonin in dogs. In all blank matrix samples, At the retention times of melatonin and IS, there were no endogenous compounds' peaks that could have caused any significant interference. The detected peak in plasma collected the day before administration accounted for less than 20% of the lower limit of quantification area. There was no carryover after upper limit of quantification injection.[46]

Ishizaki, et al. (2017) developed a sensitive, simple and rapid method to estimate melatonin using SPME equipped with LC-MS/MS. Chromatographic separation was done within 3 min by using an Inertsil ODS-3

column. This method was successfully applied to the analysis of saliva samples without any charcoal Peaks in pretreatment and interference. The reported method was applied to estimate the melatonin changes levels in saliva associated with lifestyle changes and light stimulation. This method can be considered as a helpful tool for the evaluation of sleep disorders and stress.[45]

M. van Faassen et al. (2017) reported a correlation between cortisol and melatonin in saliva and plasma. This is the primarly reported LC-MS/MS technique for comparison of melatonin in saliva and melatonin in plasma. The validation LC-MS/MS method for measuring cortisol and melatonin levels in healthy adults' free, total saliva and plasma. The results for saliva and plasma with respect to cortisol and melatonin were found to be satisfactory. Saliva collection through Salivette or passive drooling showed no significant difference; A substantial difference between salivary and free plasma melatonin (average, 36% higher), with salivary melatonin levels being, on, suggests that potential melatonin production in the salivary glands.[44]

Cristina Domenech-Coca, et al. (2019) reported an accurate analytical procedure for the assessment of the melatonin, testosterone, triiodothyronine, thyroxine and cortisol in serum. The samples of rat serum were extracted by using LLE and quantified by using HPLC coupled with tandem MS and separation was done by using MPA – 0.1% formic acid in Water and MPB – 0.1% formic acid in methanol. The established method has been used to examine the changes of testosterone, melatonin, thyroxine, cortisol and triiodothyronine in serum of rat by induced the light exposure.[43]

The application of this method holds high clinical significance as it enables the simultaneous assessment of hormones with dissimilar chemical structures, such as cholesterol derivatives and amino acid for the mood disorders and depressive illnesses.

Sunghwan Shin, et al. (2021) reported a method in saliva for estimation of melatonin and cortisol in single run & differentiated this method with immunoassays. SPE was used for the preparation of samples and analyzed using 2 mmol L^{-1} ammonium acetate in water in MPA and acetonitrile with 0.1% formic acid in MPB and Chromatographic separation was done on XDB C-18 column. Compared with the immuno assays method, HPLC coupled with tandem MS method provides more reliable and more sensitive in the estimation of melatonin and cortisol in saliva.[42]

A newly developed high-throughput sensitive bio analytical validation method was reported by Weiqi Jin et al. (2021) which is sensitive, simple, fast and high-throughput for measuring melatonin in human milk and gives an appropriate platform to quantify human milk melatonin in large scale studies. This quantification may be very useful for the development of the infant mil formula who cannot be breastfed. This quantification method can be a reference gold standard for the human clinical research implying quantification of melatonin detection in milk samples.[41]

Estimation of 19 analytes simultaneously was validated by using HPLC coupled with tandem mass spectrometry published by Anaelle Monfort et al. (2021) and it successfully applied to samples of nursing women. This method is very useful due to its simple extraction method, allowing the measuring of multiple drugs over a 1:1000 x concentration range.[40]

Demeuse J. et al. (2021) published a sensitive procedure in saliva for the estimation of melatonin. It allows the estimation at day time with a high accuracy and selectivity. The experiments showed a recovery rate of 70% and matrix factor of over 90% for the analyte. Inter-run and intra-run accuracies ranged from 89% to 113%, and the Coefficient of Variations for both were between 1% and 7%. The limit of quantification is the lowest concentration at which it was possible to easily integrate the qualitative transition's peak was found to be 0.78 pg mL^{-1}.[39]

Philippe J. Eugster et al (2022) first time reported a procedure for the detection of serotonin and its main metabolites included melatonin in human plasma by using HPLC coupled with tandem MS. Samples extracted by using protein PP followed by solid phase extraction. Improved sensitivity allows the detection of serotonin and its metabolites including melatonin even at their lowest day time concentrations of 2.2 nM for serotonin to 1.0 pM for 6-OH-Mel.[35]

Minhui Zhu et al. (2022) reported a LC-MS/MS method for the accurate measurement of melatonin, glucocorticoids and its metabolites in human hair. The method was also aimed to investigate how the concentrations of these biomarkers are related to sleep state. The developed method can quantify six endogenous compounds in hair, including cortisol and cortisone, which are important stress biomarkers. While LC-MS/MS methods exist for other sample types, such as saliva, blood and urine, there is limited research on detecting melatonin in human hair. This developed LC-MS/MS method can simultaneously quantify melatonin, N-acetyl-serotonin, 6-O-melatonin, cortisol, and cortisone in human hair. The method had a convenient hair sample pretreatment procedure and high sensitivity with limit of quantification ranging from 0.1 pg/mg for melatonin to 1.0 pg/mg for cortisol and cortisone. Analysis of 65 undergraduates' hair samples revealed relationship between poor sleep quality and higher hair cortisone content. Further investigation of hair cortisone as a biomarker for long-term sleep state in a larger sample is warranted for future studies.[55]

Dermanowski et al. (2021) developed a method for the measurement of melatonin in plasma and saliva. Further a comparison under dim light for the measurement of melatonin was carried out, since it is perceived as the most precise objective bio-marker for determining the circadian phase. The LC-MS/MS validation was done as per the European medicine agency recommendations. From twenty-one volunteers between the ages of 26 and 54 plasma and saliva samples had been taken at five different times (between 8:00 PM and 12:00 AM). Melatonin concentration was measured by using LC-MS/MS. Dim light melatonin onset was defined as the time at which melatonin concentration was recorded in plasma and saliva surpassed 7 pg/mL and 20 pg/mL, respectively. The saliva/plasma melatonin concentration ratio was 2.87, and the correlation between plasma and salivary melatonin concentration was $r = 0.764$ ($p < 0.001$). Finally, the validated method of salivary melatonin determination enabled the dim light melatonin onset assessment with potential clinical implications in monitoring and diagnosing circadian rhythm disorders.[57]

Over the years the melatonin bioanalysis has evolved significantly. In the late 90s the achievable quantification limits were in ng/mL however due to significant advancements in MS technologies pg concentrations are being achieved comfortably. Earlier the quantification matrix being used was plasma or serum only even though the collection procedure for these matrices is being painful. However, with the advancement in instruments and better sensitivities, the pain-less source of matrices e.g., hair samples can also be used these days. Also, the extraction procedures have evolved significantly from protein precipitation, liquid- liquid extraction to SPE to SPME with better recoveries and least solvent wastage. In the upcoming years, bio analysts can witness the development of fully automated estimation with minimal human intervention for better reproducibility.

CONCLUSION

Melatonin research is expected to advance significantly through the use of LC-MS/MS, a powerful analytical technique for precise and sensitive measurement of melatonin in biological samples. LC-MS/MS's capability to selectively detect and quantify melatonin in complex biological matrices, such as blood, urine, and saliva, offers the potential for more accurate measurements, even at low concentrations. This heightened sensitivity and selectivity may open up new opportunities for studying the diurnal rhythm of melatonin, investigating its physiological and pathological roles, and evaluating its therapeutic potential**.** This review describes various bio analytical methods developed and validated to estimate melatonin in various matrices such as serum, plasma, milk and saliva etc. The summary of review articles would help in the preparation of the latest bioanalytical methods. This review article presents various developed bioanalytical methods by using LC-MS/MS which covers key aspects of internal standard, sample extraction, mobile phase, CC range and instrument used. The application of LC-MS/MS has enabled tremendous quantitative capabilities in MRM mode with high sensitivity, selectivity and specificity. The usage of gradient programs helped in separation and quantification of drugs to prevent the matrix enhancement/suppression from the matrix compounds and to remove the analyte carry over from injection to injection. LC-MS/MS is a rugged and dependable instrument and it is extensively used for the quantification of melatonin because of its

shorter run time, sensitivity and selectivity. The review article provided a survey of various methods and its conditions to start new method development for melatonin.

Conflicts of interest

The authors declare no conflict of interest.

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