BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF MELATONIN IN HUMAN PLASMA BY USING LC-MS/MS

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Abstract:

Background: A hormone made by the pineal gland (tiny organ near the center of the brain). Melatonin helps control the body's sleep cycle, and is an antioxidant. It is also made in the laboratory and sold as a supplement.

Objectives: A rapid and sensitive analytical method based on liquid chromatography coupled to tandem mass spectrometry detection with ESI positive mode (Turbo spray) was developed for the determination of Melatonin in human plasma using D_4-Melatonin as the internal standard (IS).

Materials and Methods: A Solid Phase Extraction (SPE) was performed using acetonitrile. The analyte and IS were subjected to chromatographic analysis on an Eclipse XDB C_{18} (100 mm, 4.6 mm, 3.5 µm) using ACN:0.1% FA in water (80:20) as the mobile phase at a flow rate of 0.600 ml/min. An AB Sciex API 4000 Triple Quad LC/MS was operated in the multiple reaction monitoring modes. The precursor-to-product ion transitions as 232.80/173.9 (m/z) (Melatonin) and 237.10/177.90 (m/z) (D_4-Melatonin, IS) were used for quantitation.

Results: The results were linear over the studied range (52 to 5176pg/mL) and the total analysis time for each chromatograph was 1.81 ± 0.01 min. The Between run & within run Precision was range between 2.7 and 8.5 %., and the accuracy was within 117.42%.

Conclusion: This method can be used for the pharmacokinetics study of melatonin in human plasma.

Keywords: Melatonin, Bio-analysis, LC-MS/MS, Validation.

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INTRODUCTION

Melatonin is chemically (N-acetyl-3-(2-aminoethy l)-5-methoxyindole) a naturally occurring hormone that is primarily produced by the pineal gland in the brain. It is involved in regulating the body's circadian rhythm, which is the internal biological clock that controls the sleep-wake cycle[1]. Melatonin is also thought to have other important roles in the body, including regulating blood pressure, body temperature, and immune function[2]. Melatonin supplements are commonly used as a sleep aid, particularly for people with insomnia or jet lag. They are also sometimes used to help treat certain medical conditions, such as sleep disorders, depression, and anxiety[3]. In recent years, there has been growing interest in the potential health benefits of melatonin, including its role as an antioxidant and anti-inflammatory agent. Some studies have suggested that melatonin may have a protective effect against certain diseases, including cancer and cardiovascular disease[4]. Overall, melatonin is a fascinating hormone with a range of potential health benefits. As research into its effects continues, we may learn more about its potential uses and applications in the treatment and prevention of various health conditions.

Melatonin is a relatively small molecule with a simple structure, making it amenable to analysis by chromatographic methods. Chromatography is a powerful analytical technique used for separating and identifying components in a mixture based on their physical and chemical properties. The analysis of melatonin from human plasma can be challenging due to the low concentration of melatonin in plasma and the presence of interfering substances that can affect the accuracy and specificity of the analysis. The challenges involved in analyzing melatonin from human plasma are due to the low concentration (a range of 10 -100pg/mL), and interference from lipids, proteins, and other hormones that leads to inaccurate results.

The concentrations of melatonin in various biological fluids can be determined using several different analytical methods[5–16]. These include automated solid-phase extraction[10], high-performance liquid chromatography coupled with fluorescence detection[11], and high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS)[5–10] in various biological matrices like dog's plasma, cow's milk, human urine, and saliva; direct sample injection to ESI-MS/MS[13], HPLC-PDA[15], supercritical fluid chromatography coupled with tandem mass spectrometry[16]; and recently, simultaneously determining melatonin with its metabolites like 6-hydroxy melatonin in human urine by LC-MS[9]. The determination of melatonin concentration in human plasma is limited.

A quick response analysis of melatonin in human plasma is now requested by pharmaceutical companies for pharmacokinetic investigations. To the best of our knowledge, there hasn't been any research published on the use of the LC-MS/MS method to determine Melatonin in human plasma. Subsequently, a simple, efficient, and suitable method for melatonin determination in human plasma is required.

The purpose of the current research is to develop a new bioanalytical method to evaluate the concentration of melatonin in human plasma using solid-phase extraction and quantify it by LC-MS/MS. Melatonin-D_4 was employed in this method as an internal standard to ensure the accuracy of the extraction efficiency for sample preparation.

MATERIALS AND METHODS:

Chemical and Reagents: For method development, HPLC-grade chemicals and reagents were employed. Melatonin, N-[2-(5-methoxy-1H-indol-3-yl) ethyl] (Figure 1) and Melatonin D4, N-[2-(5-methoxy-1H-indol-3-yl) ethyl] (Figure 2), as an analytical and internal standard, were supplied by Vivan Life Science Pvt. Ltd., Mumbai. Blank human plasma standards were bought from Clinvend Clinical Research Solution in Hyderabad.

Additionally, HPLC-quality acetonitrile, water, and formic acid were bought from Fischer Scientific and Rankem Laboratories. The method development required any additional chemicals that were suitable for HPLC analysis.

Figure.1: Structure of Melatonin

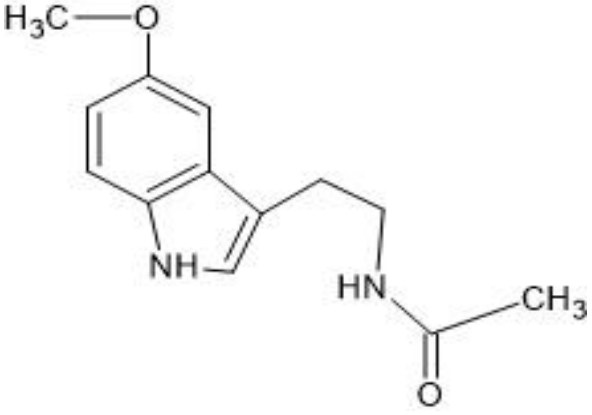
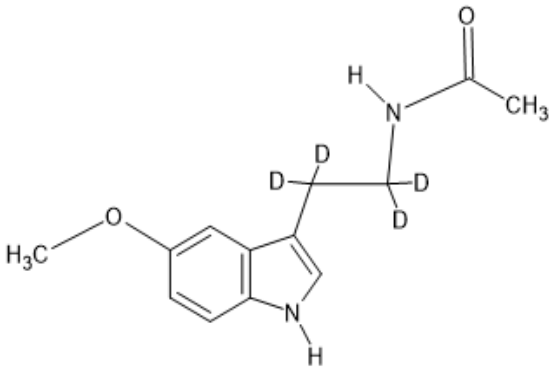


Figure.2: Structure of Melatonin-D_4



Instrumentation: For the current study, a Liquid Chromatographic (LC) system from Shimadzu, Japan, coupled with a tandem mass spectrometer (AB Sciex API 4000), a binary LC-20AD prominence pump, a CTO-20AD oven for the column, and a high-throughput SIL-20AD autosampler, has been used. Acetonitrile: 0.1% Formic Acid in water as the mobile phase (ratio - 80:20) and a flow rate of 0.600 ml/min were used to achieve chromatography on an Eclipse XDB C_{18} (4.6×100 mm, 3.5 µm). For the duration of the three-minute run, melatonin and the internal standard for melatonin-D_4 were separated. Temperatures for the analytical column and autosampler were kept monitored at 15.0°C and 45.0°C, respectively.

The Electro Spray Ionization (ESI) source, which uses a positive ionization method, received the liquid chromatographic system's eluents. To prevent unwanted contaminants from the various salts present in the human plasma samples, eluent from the chromatographic system was avoided beginning at 0.5 minutes. The mass system was programmed to the following parameters: Curtain gas: N_2 at 15 psi; Gas1: N_2 at 45 psi; Gas2: N_2 at 50 psi; temperature of the ion source, 300 °C. On MRM, the parent and product ions for Melatonin and Melatonin-D_4 were performed at 232.80/173.9 (m/z) and 237.10/177.90 (m/z), respectively. The mass conditions were presented in Table 1.

Table 1: Mass parameters for Melatonin and internal standard

Component	Parent ion	Product ion *(m/z)*	CXP (V)	DP in V	EP (V)	CE (V)
Melatonin	*m/z* 232.80	*m/z* 173.90	15	65	10	19
Melatonin-D_4	*m/z* 237.10	*m/z* 177.90	15	69	10	19

Sample Preparation: Solid Phase Extraction, To 50 µl of Melatonin-D_4 (1µg/ml) was mixed and vortexed in order to aliquot 100 µl of plasma samples. 200 µl of 20 mM Na_2HPO_4 in water were added to the resultant solution and vortexed well, then elute the sample by centrifuging the samples for 1 minute at 2500 rpm. 1 mL of HPLC-grade water and 1 mL of washing solution should be used to clean the Strata-X cartridge. At 4000 rpm, dry the Strata-X cartridge for 10 minutes. Add 0.300 mL of elution solution to the dried cartridge. Centrifuge for one minute at 2500 rpm. Transfer the eluted samples into the suitable RIA vials that have been labelled. Prepared samples should be loaded into LC-MS/MS for analysis.

Preparation of Standard Stock and Calibration Standard: Melatonin and IS stock solutions were processed in 90% methanol at concentration level of 1.0 µg/ml. Quality control (QC) and calibration standard (CC) solutions were processed by spiking blank human plasma sample from the Melatonin stock solution. CC solutions of eight different concentration levels were made to get the final concentrations of 52, 104, 244, 486, 972, 1942, 3882, and 5176pg/ml. LQC, MQC and HQC standards were prepared to produce the concentrations of 124, 1932, and 3826pg/ml, respectively. All the stock, QC and CC solutions were kept at -20°C till the method of analysis.

Validation: The method of analysis was assessed by validation parameters like specificity/ selectivity, precision, linearity, recovery, accuracy, matrix effect, and carryover test. Three QC samples of LQC, MQC, and HQCs as well as LLOQ (56pg/ml) were employed and analysed in method validation.

Accuracy and Precision: Inter-day and intra-day accuracy and precision were examined as a part of precision and accuracy (PA) parameter. Intra-day PA was evaluated by injecting QC solutions (124, 1932 and 3826pg/ml) and LLOQ (56pg/ml) in 3 replicates in a day arbitrarily. Inter-day PA was evaluated by injecting the same QC and LLOQ solutions once in a day for 3 different days. The RSDs for LQC, MQC and HQCs should be ≤20.0% for LLOQ QC and ≤15.0% for the remaining control levels.

Linearity: CC standards (Non-zero) of 8 different concentrations at 52, 104, 244, 486, 972, 1942, 3882, and 5176pg/ml solutions were prepared and

processed in 3 different runs. Linearity curve (peak area fraction of Melatonin and Melatonin D_4 peaks against original concentrations) was plotted by least squares linear regression and reciprocal of the squared concentration ($1/x^2$) utilized as a weighting factor. Deviation should be within ±20.0% for LLOQ and ±15.0% for remaining control levels.

Specificity/Selectivity: Method specificity was analysed by infusing 8 dissimilar lots of blank plasma solutions to confirm no endogenous compounds interfere with Melatonin and Internal Standard (IS). Method selectivity was analysed by equating the chromatograms acquired from blank and samples.

Recovery and Matrix Effect (ME): Melatonin recovery was assessed by paralleling the average peak response of extracted and un-extracted solutions at HQC, MQC and LQC standard levels. At each concentration level percentage recoveries was calculated and finally overall mean recovery was calculated. The ME was analysed by paralleling the un-extracted with post-extracted samples.

RESULTS AND DISCUSSION:

The LC-MS/MS peaks for the Blank, Blank with IS, Standard –A (Low) and Standard –H (High) concentration levels were shown in Figures 3-6.

Method Validation

Accuracy and Precision: Melatonin inter-day and intra-day accuracy and precision were analysed and the % RSD values were calculated for the same and were tabulated in the Table 2.

Linearity: Melatonin calibration graph was rectilinear in concentration over 52 to 5176pg/ml with regression equation of Y = 0.000593 X + 0.0163. The regression coefficient (r^2) value is more than 0.9960 which was acceptable as per the FDA regulatory guidelines.

Recovery and Matrix Effect (ME): The developed method has good recovery, the recovery values were 52.75%, 44.37%, and 41.68% for LQC, MQC, and HQC quality control samples respectively. The data for Melatonin recovery were tabulated in Table 3. The Matrix effect was evaluated at HQC and LQC level and the calculated %CV values were 0.75% and 2.02%, respectively. The data for Melatonin Matrix effect were tabulated in Table 4 and 5.

Specificity/Selectivity: By analysing eight different human plasma lots (six normal lots, one haemolysed lot and one lipemic lot) specificity and selectivity were assessed. Results were evaluated and calculated %CV values were 5.64% in Table 6.

Figure 3: Representative Chromatogram of proceed Blank Matrix

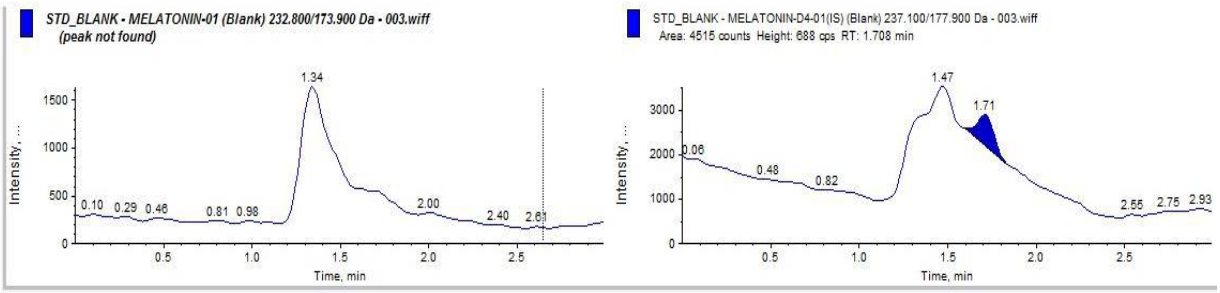


Figure 4: Representative Chromatogram of proceed Blank Matrix with Internal standard

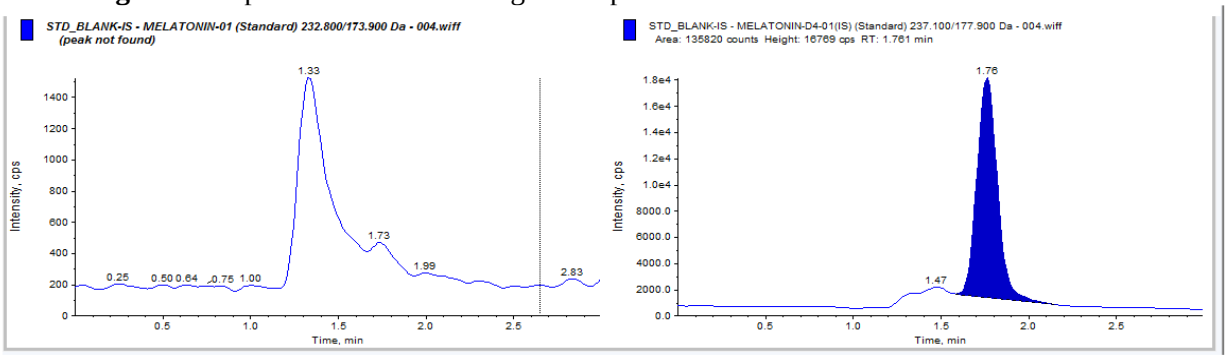


Figure 5: Representative Chromatogram of proceed Standard- A containing 52 pg/ml

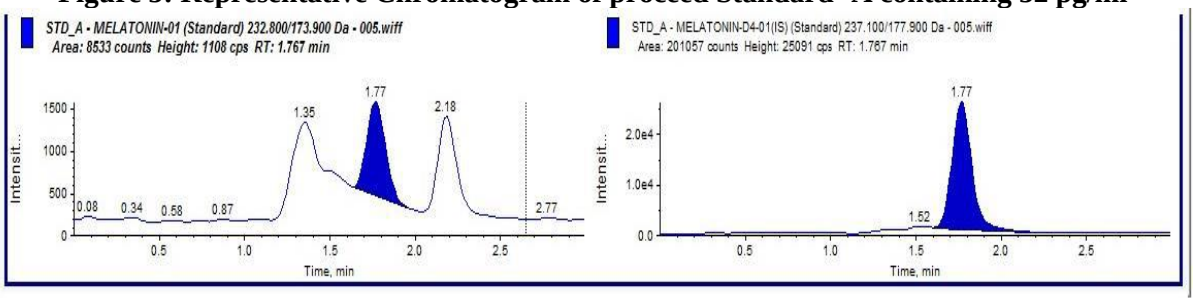


Figure 6: Representative Chromatogram of proceed Standard- H containing 5176 pg/ml

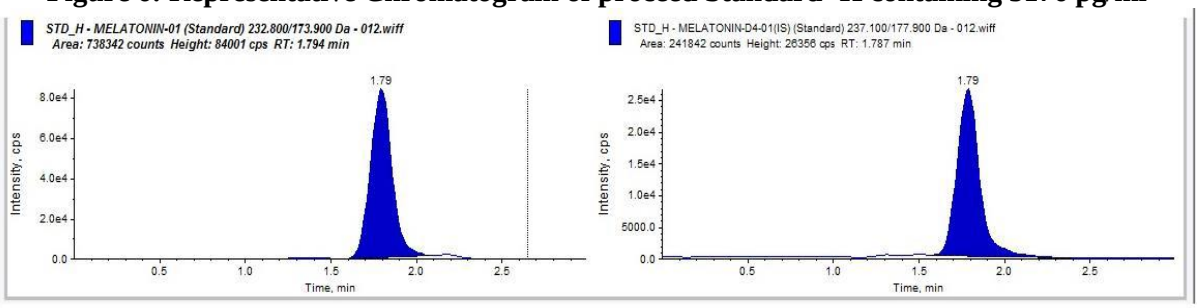


Figure 7: Representative Calibration Curve for Melatonin in matrix

Compound Name **:** Melatonin
Correlation coefficient **:** r = 0.9980, r^2 = 0.9960
Calibration Curve (y) **:** 0.000593 x + 0.0163

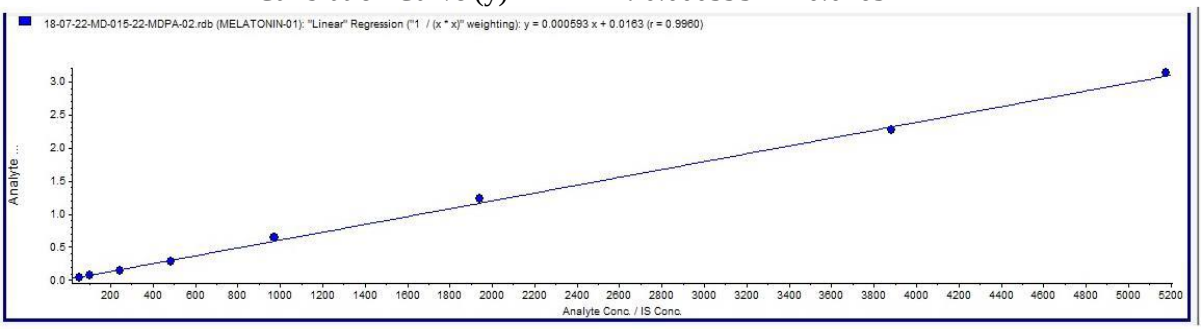


Table 2: Accuracy and Precision data for Melatonin

Nominal concentration	Intra-day		Inter-day	
	% Accuracy	%CV	%Accuracy	%RSD
52.00pg/mL	106.5	8.5	106.51	4.35
124.00pg/mL	94.42	5.0	94.42	4.06
1932.00pg/mL	92.33	5.5	92.33	4.99
3826.00pg/mL	95.59	2.7	95.93	1.46

Table 3: Recovery data for Melatonin

SL. No	LQC		MQC		HQC	
	Aqueous Analyte Area	Extracted Analyte Area	Aqueous Analyte Area	Extracted Analyte Area	Aqueous Analyte Area	Extracted Analyte Area
1	31063	17619	447925	205473	688662	358887
2	31532	18990	449606	173968	693476	235875
3	31170	16346	445479	187585	721809	287571
4	30154	14532	454123	193684	721810	292276
5	28520	13997	380751	217715	733343	333610
6	30661	15106	393751	162685	741651	284322
Mean	30517	16098	428606	190185	716792	298757
SD	1086	1928	32419	20149	21341	42802
%CV	3.56	11.98	7.56	10.59	2.98	14.33
%Recovery	52.75		44.37		41.68	

Table 4: Matrix Effect data for Melatonin (LQC)

Lot No. (LQC)	Aqueous Sample		Spiked Sample		Matrix Factor		
	Analyte Area	IS Area	Analyte Area	IS Area	Analyte	IS	IS Normalized
MAT003	42408	554620	42180	551475	1.01	1.02	0.99
MAT004	46459	601145	32379	412083	0.78	0.76	1.03
MAT005	45490	575330	33512	433047	0.80	0.80	1.00
MAT006	35512	470010	33976	456374	0.81	0.84	0.96
MAT007	39813	509998	33973	444609	0.81	0.82	0.99
MAT008	40842	538958	33658	449494	0.81	0.83	0.98
MAT009-L			35830	469105	0.86	0.87	0.99
MAT010-H			36174	483617	0.87	0.89	0.98
Mean	**41754.000**	**541676.83**				**MEAN**	**0.990**
						SD	**0.020**
						%CV	**2.02**

Table 5: Matrix Effect data for Melatonin (HQC)

Lot No. (HQC)	Aqueous Sample		Spiked Sample		Matrix Factor		
	Analyte Area	IS Area	Analyte Area	IS Area	Analyte	IS	IS Normalized
MAT003	924613	483634	1042656	533195	1.06	1.04	1.02
MAT004	952089	502349	1068784	548304	1.09	1.07	1.02
MAT005	972863	494507	1133810	583320	1.15	1.14	1.01
MAT006	918449	472881	823096	428743	0.84	0.84	1.00
MAT007	1055290	545017	873976	449666	0.89	0.88	1.01
MAT008	1073152	566361	816303	417348	0.83	0.82	1.01
MAT009-L			858087	438329	0.87	0.86	1.01
MAT010-H			718504	375080	0.73	0.73	1.00
Mean	**982742.6**	**510791.5**				**Mean**	**1.010**
						SD	0.007559
						%CV	0.75

Table 6: Selectivity/Specificity data for Melatonin

Plasma Lot ID	Specificity (Blank)		Selectivity (Spiked LLOQ)		% Interference in Blank		Area Ratio
	Analyte	IS peak	Analyte	IS peak	Analyte (<20%)	IS (<5%)	Analyte
MAT003	2317	3690	15749	319367	14.7120	1.2483	0.049
MAT004	2821	2598	16862	319726	16.7299	0.8126	0.053
MAT005	2141	1510	11483	236481	18.6450	0.6385	0.049
MAT006	3011	20111	16445	307058	18.3095	6.5496	0.054
MAT007	1110	2148	14905	288726	7.4472	0.7440	0.052
MAT008	2582	3821	17271	306567	14.9499	1.2464	0.056
MAT009	1489	3227	16260	283125	9.1574	1.1398	0.057
MAT010	1792	29567	16697	303831	10.7325	9.7314	0.055
			Mean	**295610.125**	**13.83543**	**2.76381**	**0.05313**
						SD	**0.002997**
						%CV	**5.64**

CONCLUSION:

A Simple and Specific LC-MS/MS techniques for the melatonin was developed and validated by utilizing melatonin-D_4. This method has good recovery, accuracy and precision compared with existed methods for the analysis of drug in plasma samples. The drug was subjected for extraction with acetonitrile from human plasma samples by SPE. The drug was eluted within 3mins using an Eclipse XDB (100mm, 4.6mm, 3.5µm) C_{18}-analytical column with isocratic system by ACN:0.1% FA in water (80:20) as movable phase with flow rate of 0.600ml/min. The developed technique was validated as per the FDA regulatory guidelines and all the parameters of validation were within standard limit. This method can be used for the pharmacokinetics study of melatonin in human plasma.

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ABBREVIATIONS: LC-MS/MS: liquid chromatographic-tandem mass spectrometry, SPE: Solid Phase Extraction, FDA: Food and Drug Administration; QC: quality control; SD: standard deviation, LQC: low quality control; MRM: Multiple reaction monitoring; LLOQ: lower limit of quantification; HQC: high quality control; MQC: median quality control; CV: coefficient of variation.

REFERENCES

1. Sylvie Tordjman, Sylvie Chokron, Richard Delorme, Annaëlle Charrier, Eric Bellissant, Nemat Jaafari, and Claire Fougerou, Melatonin: Pharmacology, Functions and Therapeutic Benefits. Curr Neuropharmacol. 2017 Apr; 15(3): 434–443.
2. B Claustrat, J Leston, Melatonin: Physiological effects in humans. Neuro chirurgie, 2015 Apr-Jun;61(2-3):77-84.
3. Zizhen Xie, et al., A review of sleep disorders and melatonin. Neurol Res, 2017 Jun;39(6):559-565.
4. Diana Maria Chitimus, et al., Melatonin's Impact on Antioxidative and Anti-Inflammatory Reprogramming in Homeostasis and Disease. Biomolecules. 2020 Sep; 10(9): 1211.
5. Huimin Zhao, Yifei Wang, Yi Jin, Shu Liu, Haiyan Xu, Xiumei Lu * , Rapid and sensitive analysis of melatonin by LC-MS/MS and its application to pharmacokinetic study in dogs. Asian journal of pharmaceutical sciences, 2015.
6. An-Qi Wanga, Bo-Ping Wei b, Yan Zhanga, Yu-Jun Wanga, Liang Xua, Ke Lana, 2011. An ultra-high sensitive bioanalytical method for plasma melatonin by liquid chromate graphy–tandem mass spectrometry using water as calibration matrix. Journal of Chromatography B, 879 (2011) 2259–2264.
7. Nihat Ozcan & Soyhan Bagci, 2017. Determination of Melatonin in Cow's Milk by Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS). Food Anal. Methods.
8. Tolgahan Kocadag˘lı, Cemile Yılmaz, Vural Gökmen, 2013. Determination of melatonin and its isomer in foods by liquid chromatography tandem mass spectrometry. Food Chemistry 153 (2014) 151–156.
9. G. Magliocco, F. Le Bloc'h, A. Thomas, J. Desmeules, Y. Daali*, 2021. Simultaneous determination of melatonin and 6-hydroxymelatonin in human overnight urine by LC-MS/MS. Journal of Chromatography B, Volume 1181, 1 September 2021, 122938.
10. Duraisamy Karunanithi & Ammanamanchi Radhakrishna & Kunnummal Parambil Sivaraman & Valsala Madhavan Nair Biju, 2013. Quantitative determination of melatonin in milk by LC-MS/MS. J Food Sci Technol (April 2014) 51(4):805–812.
11. S. Romsing, F. Bo¨ Kman & Y. Bergqvist, Determination of melatonin in saliva using automated solid-phase extraction, high-performance liquid chromatography and fluorescence detection. Scand J Clin Lab Invest 2006; 66: 181–190.
12. Erik Bechgaard, Karsten Lindhardt, Lise Martinsen. 1998. High-performance liquid chromatographic analysis of melatonin in human plasma and rabbit serum with on-line column enrichment. Journal of Chromato graphy B, 712 (1998) 177–181.
13. Shuming Yang, Xiaohui Zheng, Yan Xu,*,Xiang Zhou, 2002. Rapid determination of serum melatonin by ESI–MS–MS with direct sample injection. Journal of Pharmaceutical and Biomedical Analysis 30 (2002) 781–790.
14. Leiziani Gnatkowski Martins, Najeh Maissar Khalil, Rubiana Mara Mainardes∗, 2017.
15. Application of a validated HPLC-PDA method for the determination of melatonin content and its release from poly(lactic acid) nanoparticles. Journal of Pharmaceutical Analysis 7 (2017) 388–393.
16. Denise Wolrab, Peter Frühauf, Christopher Gerner*, 2016. Quantification of the neurotransmitters melatonin and N-acetyl2 serotonin in human serum by supercritical fluid chromatography 3 coupled with tandem mass spectrometry. Analytica Chimica Acta (2016).