A rapid and sensitive High-Performance Liquid Chromatography method with fluorescence detection for quantification of melatonin in small volume rat plasma samples: application to a preclinical study to determine the oral pharmacokinetics of melatonin under gestational conditions

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A novel, simple and sensitive high-performance liquid chromatography with fluorescence detection method was developed and validated for the characterization of the preclinical pharmacokinetics of melatonin under pregnant conditions. Plasma samples (25 µL) were treated with 30 µL of ethanol absolute (containing the internal standard, IS). After a centrifugation process, aliquots of supernant (5 µL) were injected into the chromatographic system. Compounds were eluted on a Xbridge C18 (150 mm x 4.6 mm i.d., 5 µm particle size) maintained at 30°C. The mobile phase consisted in a mixture of aqueous solution of 0.4% phosphoric acid and acetonitrile (70:30 v/v). The wavelengths were set at 305 nm (excitation) and 408 nm (emission) and the total analysis time was 8 min/sample. All validation tests were obtained with accuracy and precision, according to FDA guidelines, over the concentration range of 0.005-20 µg/mL. Pharmacokinetic study showed that melatonin systemic exposure increased from day 14, with a significant difference at 19 days of gestation compared to the control group. Our findings suggest a decreased metabolism of melatonin as result of temporary physiological changes that occur throughout pregnancy. However, other maternal physiological changes cannot be ruled out.

Keywords: Melatonin. High-Performance Liquid Chromatography. Pharmacokinetics. Gestation. Rats.

INTRODUCTION

The pineal hormone melatonin has shown to be a promising pleiotropic molecule with diverse multi-regulatory functions, such as: the regulation of the

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circadian rhythm, sleep quality, blood pressure, and body temperature (Wetterberg, 1999; Zisapel, 2018). Furthermore, broad potential therapeutic properties have been reported through the exogenous administration of melatonin in preclinical studies and clinical trials, including anxiolytic, immunomodulatory, antioxidative, antiapoptotic and antinociceptive activities; as well as therapeutic utility in the management of viral infections (Anderson, Reiter, 2020; Arreola-Espino *et al.*, 2007; Hemati *et al.*, 2021; Miguel *et al.*, 2022; Roy *et al.*, 2022; Xu *et al.*, 1996; Zisapel, 2018). Moreover, melatonin

may have an important role in maintaining a healthy pregnancy, this has been demonstrated in different preclinical models as well as in pregnant women (Vine, Brown, Frey, 2022; Tamura *et al.*, 2008), which makes this compound an important therapeutic option to be considered for exogenous administration during pregnancy in deficiency circumstances.

It has been described that most of melatonin therapeutic effects depend on the administered doses (Di *et al.*, 1997). To our knowledge, the range of effective doses studied in different preclinical models varies from 1 to 500 mg/kg, whereas in clinical trials the reported dose range is between 0.5 to 100 mg; these depending on the clinical indications or research purpose (Arreola-Espino *et al.*, 2007; Carloni *et al.*, 2017; Choudhary *et al.*, 2019; Hendawy *et al.*, 2021; Nickkholgh *et al.*, 2011). This wide range of effective doses may be attributed to several pharmaceutical and physiological issues, such as: (i) pharmaceutical formulation, (ii) vehicle employed for the preparation of oral solutions or suspensions (due to limited solubility of melatonin in water), (iii) its poor and variable oral bioavailability as result of an extensive first pass effect in the liver by the CYP1A2 enzyme and (iv) the physiological or pathological conditions of the organism (Andersen *et al.*, 2016; Cheung *et al.*, 2006; Choudhary *et al.*, 2019; Di *et al.*, 1997; Harpsøe *et al.*, 2015; Härtter *et al.*, 2000; 2001; Moroni *et al.*, 2021; Yeleswaram *et al.*, 1997). All of these may cause inconsistent pharmacokinetic results as well as compromise the understanding of its pharmacokinetic-pharmacodynamic correlation (Chen, Stone, 2019). In that sense, it has been suggested as an important consideration, the detailed investigation of the pharmacokinetics of melatonin in preclinical models before translation to clinical use (Cheung *et al.*, 2006; Choudhary *et al.*, 2019). Since the therapeutic use of melatonin during pregnancy is just emerging and considering that pregnancy is associated with a multitude of temporal physiological and metabolic changes that can alter maternal drug disposition in different ways through the gestational periods, as it has been reported for the expression and activities of drug-metabolizing enzymes including CYP1A2 (Anderson, Carr, 2009; Gaohua *et al.*, 2012; Ke *et al.*, 2014; Yu *et al.*, 2016), these alterations may impact the pharmacokinetics

of melatonin and therefore, its therapeutic range. One of the objectives of this study was to characterize the oral pharmacokinetics of melatonin in different gestational stages and compare it with nonpregnant controls, using rats as preclinical model.

For that purpose, it is essential to employ well-characterized and fully validated bioanalytical methods to yield reliable results which can be satisfactorily interpreted (Shah *et al.*, 2000). In the case of melatonin, there are scarce bioanalytical methods for its determination in animal plasma samples after an exogenous administration. These methods include mainly the use of fluorescence or mass-mass spectrometry detection and sample preparation techniques such as: protein precipitation (Choudhary *et al.*, 2019), solid phase extraction (Yeleswaram *et al.*, 1997), or a combination of techniques with liquid-liquid extraction (Zhao *et al.*, 2016). However, these methods have certain characteristics that may limit their use, such as: the use of mobile phase gradients, several extraction steps, a scarce sensitivity, or the use of high-cost instrumentation such is LC-MS/MS, which is not easy to acquire and/or to adapt in many laboratories.

Considering the need for more simple, sensitive, and low-cost bioanalytical methods to determine melatonin in preclinical testing, the main purpose of this work was to develop and validate a reliable HPLC-FL micro-assay to determine this compound after its exogenous administration in rats.

MATERIAL AND METHODS

Animals

Female nonpregnant and pregnant Wistar rats (250-300 g) were kept under controlled temperature and relative humidity conditions and maintained on a 12-h light/dark cycle. All the animal handling procedures for this study were carried out according to the corresponding Mexican Official Norm NOM-062-ZOO-1999 as well as considering the principles of the 3Rs: replacement, reduction, and refinement (NC3Rs, 2020). Additionally, these were previously reviewed and approved by the National Institute of Perinatology Research Animal Care (CICUAL), Biosecurity and Research committees

(protocol approval numbers: 2020-1-17 and 2021-1-16). All experiments were carried out under fasting conditions (12 h) and the animals had free access only to drinking water. Rats were used only once, and efforts were made to minimize animal suffering. Additionally, the number of rats used was the minimal required to obtain significant statistical power (NC3Rs, 2020).

Chemicals and reagents

Melatonin and salicylic acid (internal standard, IS) reference standards (Figure 1) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the United States Pharmacopeia; respectively. Melatonin (raw material) was kindly gifted by Productos Medix, S.A. de C.V. (Mexico City, Mexico). Acetonitrile (HPLC grade) and phosphoric acid (analytical grade) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Ethanol absolute of analytical grade was purchased from Reactivos Química Meyer (Mexico City, Mexico). Deionized water was obtained through an Easy Pure system (Waters Inc., Milford, MA, USA). Drug-free rat plasma was obtained from the National Institute of Perinatology bioterium.
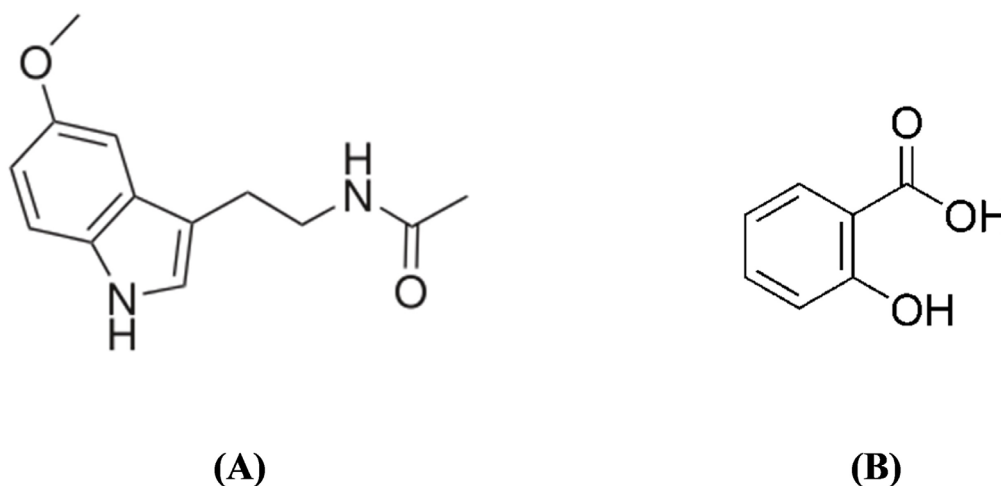

(A)   **(B)**

FIGURE 1 - Chemical structure of melatonin (A) and IS (salicylic acid) (B).

Instruments and conditions

HPLC analyses were carried out on a Waters e2695 Separations Module and a model 2475 Multi λ fluorescence detector. Data were recorded using Empower software (from Waters Assoc, Milford, MA, USA). The analytical column employed was a Waters XBridge® C18 column (150 x 4.6 mm, i.d., 5 μm) which was kept at 30°C. Mobile phase consisted of a mixture of 0.4% phosphoric acid and acetonitrile (70:30 v/v) and was filtered through a 0.45 μm cellulose membrane filter and degassed before use. Flow rate was 1.1 mL/min. Wavelengths were 305 nm (excitation) and 408 nm (emission). The autosampler temperature was set to 10°C, the injection volume was 5 μL and the run time was 8 min per sample.

Preparation of standards and quality controls

Melatonin and IS were dissolved in absolute ethanol to concentrations of 1 mg/mL and were stored at -20°C. Working standard solutions of melatonin were prepared by diluting the stock standard solution with the same solvent to yield eight calibration standards from 0.1 to 400 μg/mL. These concentrations were in considering our national regulatory guideline for the validation of bioanalytical methods, where the added volume of these solutions to be dissolved in plasma must not be greater

than 5% of the final volume of the sample (Mexican Official Norm NOM-177-SSA1-2013). Thus, the different working solutions (50 µL) were added to drug-free plasma (950 µL) in bulk to obtain melatonin plasma concentration levels of 0.005, 0.01, 0.025, 0.1, 0.5, 2.5, 5, 10 and 20 µg/mL for calibration curve samples. Likewise, quality control samples were also prepared in bulk at four levels (in addition to lower limit of quantification (LLOQ)) over the concentration range used for calibration: 0.015 (low), 8 (medium) and 16 µg/mL (high). The IS solution was prepared in absolute ethanol at a concentration of 15 µg/mL and kept at -20°C to be used for protein precipitation during the sample preparation procedure.

Sample preparation procedure

An aliquot of 25 µL of plasma sample was placed in a 0.5 mL polypropylene conic tube with a snap-on cap. Then, plasma protein precipitation was carried out by addition of 30 µL of cold IS solution. After, the sample was vortexed for 30 s at maximal speed and centrifuged at 10,000 rpm for 5 min at 10°C. The upper layer (≈30 µL) was transferred to a clean chromatographic vial and aliquots of 5 µL were injected into the chromatographic system.

Method validation

In order to confirm the suitability of this bioanalytical method for its intended use, it was fully validated according to the guidelines of the US Food and Drug Administration (FDA, 2018). Validation included selectivity, calibration curve, accuracy and precision, extraction recovery, sensitivity (LLOQ), stability and dilution integrity tests. Accuracy and precision of the method were considered as acceptance criteria. Accuracy was calculated using the formula ((measured value – theoretical value) / theoretical value) x 100; whereas assay precision was assessed by expressing the coefficient of variation of the measurements as a percentage. Accuracy was ± 15% of the nominal concentration and precision did not exceed 15%, except at LLOQ where the accuracy of the calibrator was ± 20% of the nominal concentration and its precision did not exceed 20%.

The selectivity of the method for endogenous interferences was evaluated by comparing chromatograms of pooled blank rat plasma samples with blank plasma spiked with melatonin and IS, as well as plasma samples obtained after oral administration of melatonin in rats.

The calibration curve was determined by plotting the peak-area ratios (melatonin/IS) versus the melatonin concentrations in a range from 0.005 to 10 µg/mL and was used to assess linearity using least squares regression analysis with data obtained from at least three runs. The best regression model was chosen after exploration of different models and weighting factors. The sensitivity of this method was evaluated by the measurement of LLOQ (0.005 µg/mL), which must be at least 5 times the response compared to the blank response. The accuracy and precision were determined from five replicates on the same day and in at least three runs. To evaluate the accuracy and precision intra- and inter-day, five replicates of spiked QC samples (at three levels: low (0.015 µg/mL), medium (8 µg/mL) and high (16 µg/mL) as well as the LLOQ were analyzed on the same day and similarly in three consecutive runs, respectively. The absolute recovery was calculated with QC samples by comparing the peak areas of melatonin in the spiked plasma samples with plasma-free samples containing the same amount of melatonin. The extraction recovery of the IS was determined in a similar way. The stability of melatonin was evaluated on QC samples at low and high concentration levels. The QCs samples were analyzed after the applied experimental conditions: bench-top for 3 hours; autosampler for 24 hours; long-term storage at -20°C for 17 days, 24 hours after extract processing at 2-8°C; stock and working solutions stored at 2-8°C for 30 days. During stability testing all samples were analyzed in triplicate and compared versus freshly prepared samples. Finally, dilution integrity test was performed for the high-level QC by 1:2 dilution in five replicates, with screened rat blank plasma.

Application of the validated method in pharmacokinetic studies

Previous to its application in the pharmacokinetic study under gestational conditions in rats, we decided to

demonstrate the preclinical usefulness of this method with a pilot pharmacokinetic study of melatonin administered in different doses in Wistar rats.

A total of eight male Wistar rats (250-300 g) (provided by the National Institute of Perinatology bioterium and maintained on a 12-h light/dark cycle) were randomly and proportionally grouped into four groups. On the day of experiment, rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and a polyethylene catheter (a combination of PE-10 and PE-50 cannulas, Clay Adams, Parsippany, NJ, USA) was inserted into the caudal artery through a surgical implant for blood sampling. The catheter was kept patent with heparinized saline solution and stopped with a needle. A dosing formulation of melatonin was prepared considering an administration of 5 mL/kg of melatonin, which was dissolved in 10% ethanol prior to administration. Groups received a single dose, by oral gavage, of 2.5, 5, 10 and 20 mg/kg of melatonin. Serial blood samples (100 µL) were collected before dosing and 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 240, 360 and 480 min after compound administration. Plasma samples were obtained by centrifugation at 3500 rpm for 10 min at 4°C and stored at -20°C prior to analysis. All samples were analyzed within 1 week after the pharmacokinetic study.

For the pharmacokinetic study under gestational conditions, a group of nonpregnant and pregnant rats were randomly divided into four groups (n=3; each): control (nonpregnant), 7 (G7D), 14 (G14D) and 19 (G19D) days of gestation. In the day of experiment, rats were handling under similar procedures and conditions of the pharmacokinetic study mentioned above. Animals received a single oral dose of 20 mg/kg of melatonin and serial blood samples (100 µL) were collected before dosing and 5, 10, 15, 30, 45, 60, 90, 120, 180, 240 and 360 min after compound administration. Plasma was obtained and stored as mentioned above.

Pharmacokinetic analysis and statistics

Individual plasma concentrations against time curves were constructed. Maximum concentration (C_{max}) and time to reach this maximum (t_{max}) were determined directly from these graphs. Area under the plasma concentration-time curves (AUC_{480min}) were obtained by the trapezoidal method (Rowland & Tozer, 1989). Terminal half-life ($t_{1/2}$) was obtained by log-linear regression of the terminal decay phase. Apparent oral clearance (Cl/F) and apparent volume of distribution (Vd/F) were obtained by non-compartmental techniques. All pharmacokinetic analyses were carried out using WinNonlin® Professional software, version 2.1. All calculated pharmacokinetic parameters were expressed as arithmetic mean ± standard deviation (SD). For the statistical analysis, pharmacokinetic parameters were analyzed by one-way analysis of variance (ANOVA) with Tukey's test for post-hoc comparison and using SigmaStat® software version 4.0. Statistical significance was achieved when $p < 0.05$.

RESULTS AND DISCUSSION

Method development

We present a new method for determination of melatonin in small samples of rat plasma. The assay method was focused on development and optimization of suitable small plasma sample preparation, sensitive detection and chromatographic separation of melatonin using a simple isocratic elution. Fluorescence detection was used because it is highly selective and sensitive in comparison with UV detection. This was very useful for measuring melatonin in small volume of plasma under the experimental design used in this study. Moreover, the used instrumentation is less expensive in comparison with the electrospray ionization - tandem mass spectrometry detection, which is not commonly available in most laboratories in developing countries.

Our assay offers some advantages over the previously reported bioanalytical methods for determination of melatonin in animal plasma samples after its exogenous administration (Cheung *et al.*, 2006; Choudhary *et al.*, 2019; Yeleswaram *et al.*, 1997; Zhao *et al.*, 2016). First, we were able to resolve the issue of the IS selection adequately. Often it is difficult to identify a commercially available fluorescing IS, which elutes within a reasonable time after the principal analyte. However, we found in salicylic acid a suitable

compound that met with these features, and it offered relatively high and reproducible recovery. In addition, this compound can be easily purchased at a relatively low cost. A possible disadvantage of this compound is that it is the main metabolite of aspirin, which is an over-the-counter (OTC) drug. However, for the preclinical purpose for which this method was designed, this is not critical (Srinivas, 2016). Second, we used mobile phase containing an aqueous component that is easily prepared, without the need for additional instruments (balance and pH-meter). In addition, the mobile phase components ratio was successfully determined in order to have a constant flow rate obtaining a good selectivity, a short run time and an optimal range of column pressures (around 1600 psi); which can help extend the lifespan of the chromatographic column, without affecting the resolution, the peak shape and the chromatographic responses of melatonin and the IS.

Another feature that makes this method a good alternative for melatonin determination in rat plasma after its exogenous administration is that it requires only one-step extraction procedure by protein precipitation, using only 25 µL of plasma sample and 30 µL of the precipitant reagent. By using absolute ethanol as deproteinizing agent, instead of methanol or acetonitrile, melatonin and IS were easily extracted with adequate sample cleaning and high selectivity. Selecting this solvent allowed us to avoid significant sample dilution and at the same time obtain a sufficient amount of supernatant to inject 5 µL into the chromatographic system, bringing reliable results. Furthermore, ethanol is a compound with less risk to human health and more environmentally friendly solvent.

Regarding the use of small plasma samples, it is well established that the use of small or microvolumes of biological fluids is a highly valued ethical feature when conducting pharmacokinetic studies in small animals or even in clinical settings for special populations. In that sense, animal models such rats are commonly used in the pharmacological studies of melatonin to explore its therapeutic effects. In the case of preclinical pharmacokinetic studies, extraction of small volumes of blood allows for repeated sampling from the same animal, reducing the number of subjects used per study, which can help to meet regulatory requirements regarding protection of animals used in scientific and medical research (NC3Rs, 2020). Moreover, using small volumes results in a less destructive method, comparing to those with larger sample volumes. During the development of this method, efforts were made to considerably lower the required plasma volume to 25 µL, and still provide good sensitivity for quantification of melatonin. This represents a great advantage comparing to those methods that even use volumes of up to 1 mL of biological sample (Yeleswaram *et al.*, 1997).

Method validation

Blank plasma samples from six different animals including a hemolytic sample were analyzed in order to test the selectivity of the method due to the endogenous components which might interfere during the quantitative analysis of melatonin and IS. As presented in Figure 2, the chromatograms were free of interfering peaks at the retention times of melatonin (3.89 min) and IS (6.94 min). In general, the retention times were very stable with standard deviations between 0.01 and 0.02 min. In addition, there was a good resolution between the peaks of the compounds in a chromatographic run time of 8 min.
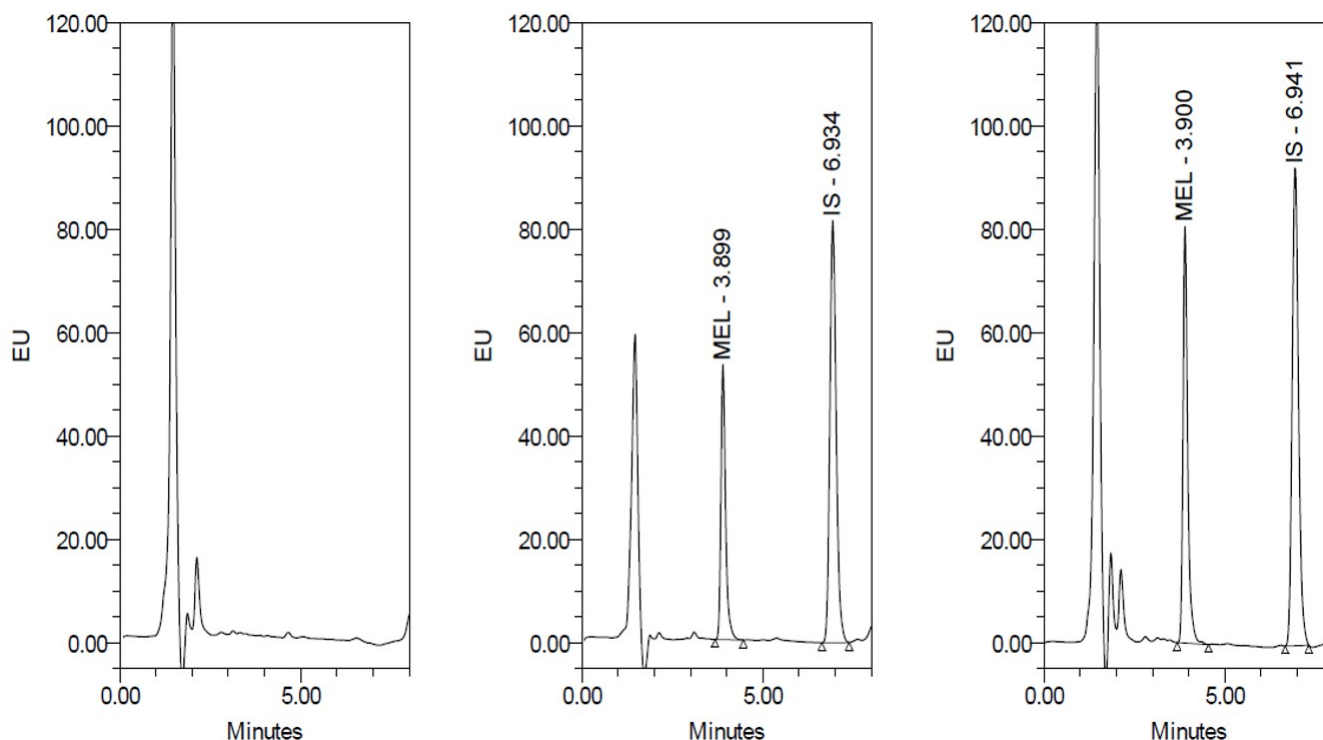
FIGURE 2 - Typical chromatograms obtained after injection of plasma extracts to the chromatographic system. (A) blank plasma; (B) sample corresponding to a point of the calibration curve (2.5 µg/mL); (C) sample obtained from a rat 60 min after a single administration of a dose of 20 mg/kg (p.o.) of melatonin (MEL).

Standard curves of melatonin were prepared in blank plasma from eight concentrations in the range of 0.005 – 20 µg/mL, including the LLOQ, by diluting standard solutions in blank plasma (n=3). The linearity was determined from the constructed standard calibration curve obtained by plotting chromatographic peak area ratios (melatonin/IS) versus the respective nominal concentration of melatonin and using a weighting factor of $1/x^2$. The calibration graphs for melatonin resulting plots followed a good linear regression in the mentioned concentration range, with mean correlation coefficient (r) of 0.9962. Optimum accuracy for the corresponding calculated concentrations at each level was obtained after performed the linear regression analysis. Moreover, small RSD values for the slopes of calibration curves were observed. All these results are shown in Table I. The lowest amount of melatonin that was quantitatively determined with acceptable precision and accuracy was 0.005 µg/mL. Results are shown in Table II.

TABLE I - Calculated concentrations of melatonin in calibration standards prepared in rat plasma (n=3)

Added concentration (µg/mL)	Measured concentration (µg/mL)	SEM	CV (%)	RE (%)
0.005	0.0049	0.0002	5.5	-2.5
0.01	0.011	0.0002	3.2	11.6
0.025	0.024	0.002	11.6	-5.2
0.1	0.10	0.001	1.4	2.6
0.5	0.48	0.012	4.2	-3.3
1	0.97	0.043	7.7	-3.3
5	4.90	0.12	4.4	-1.9
10	10.59	0.20	3.2	5.9
20	21.33	0.28	2.2	6.6
Slope	1.07	0.009	1.5	
r	0.9996	0.0001	0.1	

TABLE II - Accuracy, precision, and recovery in QC samples obtained during intra- and inter-day analysis with the method for determination of melatonin in samples of rat plasma. Data are expressed as the mean of five determinations

Nominal concentration (µg/mL)	Measured concentration (µg/mL)	SEM	Accuracy (%)	Precision (%)	Recovery (%)
Intra-day					
0.005	0.0046	0.0001	-7.0	7.1	
0.015	0.0145	0.0005	-3.0	8.6	91.0
8	7.68	0.25	4.0	7.2	
16	16.3	0.7	7.3	9.1	94.6
Inter-day					
0.005	0.0049	0.0015	-1.2	12.0	
0.015	0.0153	0.0007	2.0	11.2	
8	8.14	0.16	1.8	7.6	
16	16.5	0.4	9.1	9.5	

Method's accuracy, precision and recovery results are shown in Table II. Intra-assay accuracy and precision ranged from -7.0 to 7.3%, and from 7.1 to 9.1%, respectively. Inter-assay accuracy was between -1.2 and 9.1%, with a precision of ≤ 12.0%. The absolute recovery from the drug-spiked plasma across QC samples, when compared with equivalent aqueous samples, was within 91.0 and 94.6% with %RSD less than 7.6%. In addition, the absolute recovery of IS was 94.4%. As it can be observed, the assay exhibited satisfactory accuracy, precision, and recovery.

The stability data of melatonin in rat plasma at low and high concentrations in different conditions are shown in Table III. As shown, no significant alteration regarding the nominal concentrations of melatonin were observed. Melatonin in plasma was shown to be stable at ambient conditions (22°C) for at least 3 h, as well as after three freeze-thaw cycles of plasma samples. As for the stability of the processed for at least 24 h and the stability of the extracted analytes under the autosampler conditions for at least 24 h, they were satisfactorily determined. The long-term stability was found to be at least 17 days at the storage condition of -20°C. The stock solutions of melatonin and IS were stable at -20°C in the investigated period since the responses of these compounds were found to have a relationship from 0.94 – 1.06 of that of the freshly prepared solutions with the same concentrations.

TABLE III - Accuracy and precision obtained during the stability and dilution integrity tests of melatonin in plasma samples. Data are expressed as the mean of three determinations

Test evaluated	Nominal concentration (µg/mL)	Measured concentration (µg/mL)	Accuracy (%)	Precision (%)
Dilution integrity (1:2)	16	15.6	-2.5	11.2
Bench-top for 3 h	0.015	0.0147	-1.7	8.3
	16	17.8	11.3	4.3
Three freeze-thaw cycles	0.015	0.0148	-1.1	11.9
	16	17.4	10.8	3.1
Autosampler stability for 24 h	0.015	0.0156	3.7	8.0
	16	18.0	11.5	2.4
Extraction stability at 2-8°C for 24 h	0.015	0.0144	-3.9	9.5
	16	16.8	4.7	10.9
Long-term for 17 days at -20°C	0.015	0.0140	-6.6	11.5
	16	16.2	1.2	6.5

	Nominal concentration (µg/mL)	Area relationship (old/freshly prepared solutions)	Precision (%)	
Stock solution stability for 30 days	0.3	1.06	1.7	
	320	0.94	2.5	

Application to a pilot pharmacokinetic study

The suitability of the proposed method was proved initially in a preclinical pharmacokinetic study of melatonin. After administration, the plasma concentrations of melatonin were determined by the described method. The method demonstrated adequate sensitivity and selectivity in quantifying melatonin plasma levels after administration of a single dose of at least 2.5 mg/kg. However, it is important to mention that for this tested dose, the period of concentrations observed over the LLOQ was lower (60 min) in comparison with the other doses tested. This impacts on the pharmacokinetic characterization, since the mean

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AUCt/AUC∞ ratio was 67%, whereas for the higher doses this ratio was >99%.

Mean (±SEM) plasma concentration-time profile following an oral gavage of a single dose in the range of 2.5 to 20 mg/kg in Wistar rats was depicted in Figure 3. The profiles revealed a fast absorption of melatonin (tmax ≈ 12.5 min). Although the number of experimental subjects used in this study was low, it was possible to observe a short $t_{1/2}$ value and apparent non-linear pharmacokinetics over the range of applied doses, which is consistent with previous preclinical reports (Yeleswaram et al., 1997). The pharmacokinetic parameters were summarized in Table IV.

It is important to mention that a possible clinical application of this bioanalytical method cannot be ruled out, since it has been reported that mean peak plasma melatonin levels (Cmax) in preterm neonates after repeated melatonin administration of 1 and 5 mg/kg i.v., were found to be 1.03 and 7.05 µg/mL, respectively, which are within the calibration range evaluated in this assay (Carloni et al., 2017). Considering the ethical aspects of blood sampling for this special population, we believe that our method can be very useful, if necessary, due to the great advantage of using plasma aliquots of only 25 µL. However, this possibility needs to be evaluated in further studies.
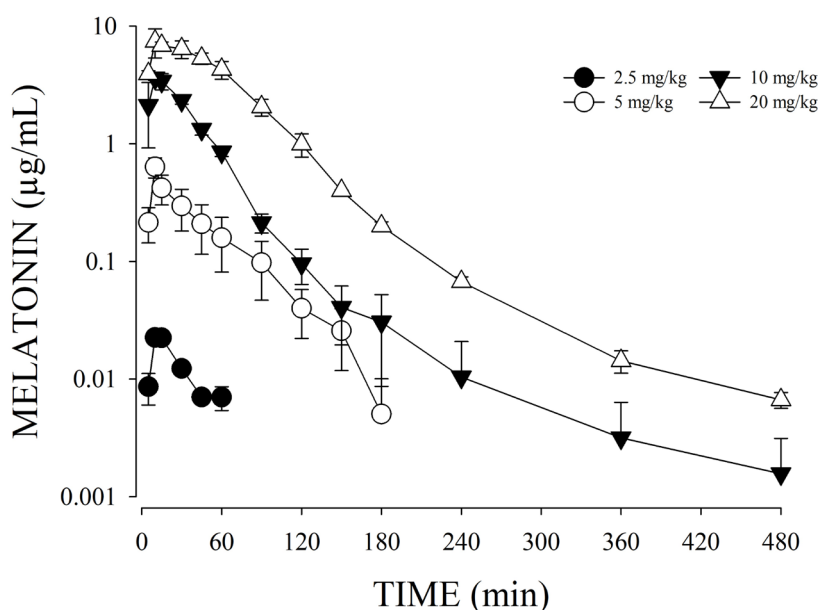

FIGURE 3 - Mean plasma levels vs time curve after the administration of a single dose of melatonin at four doses (2.5, 5, 10 and 20 mg/kg) (p.o.) in rats. Data are presented as mean (n=2) ± S.E.M.

TABLE IV - Pharmacokinetic parameters obtained after a single administration of four different doses of melatonin in rats. Data are expressed as the results of two determinations

Parameter	Dose (mg/kg)			
	2.5	**5**	**10**	**20**
$T_{max\ (min)}$	10 / 15	10 / 15	10 / 15	10 / 15
$C_{max\ (µg/mL)}$	0.020 / 0.025	0.30 / 0.76	3.4 / 3.9	6.3 / 9.5
$AUC_{last\ (µg·min/mL)}$	0.69 / 0.73	14.2 / 32.9	127.7 / 160.4	436.8 / 576.2
$AUC_{\infty\ (µg·min/mL)}$	0.94 / 1.47	14.7 / 33.2	128.03 / 160.7	437.2 / 576.6
$t_{1/2\ (min)}$	32.9 / 59.8	30.3 / 35.3	18.8 / 33.6	42.5 / 42.4

TABLE IV - Pharmacokinetic parameters obtained after a single administration of four different doses of melatonin in rats. Data are expressed as the results of two determinations

Parameter	Dose (mg/kg)			
	2.5	**5**	**10**	**20**
$C_{last\ (\mu g/mL)}$	0.0054 / 0.0086	0.012 / 0.010	0.009 / 0.006	0.006 / 0.008
$T_{last\ (min)}$	60 / 60	150 / 180	180 / 360	480 / 480

t_{max}: time to reach the maximum concentration. C_{max}: maximum concentration observed. AUC: Area under the curve. $t_{1/2}$: terminal half-life. C_{min}: minimum concentration observed. t_{last}: time of last measurable concentration

Influence of gestational stage on the oral pharmacokinetics of melatonin

We chose rats as a model due to their relatively short average gestation time (21 to 23 days). Figure 4 depicts the mean ± SEM plasma level-time course of melatonin obtained after the administration of an oral dose of 20 mg to pregnant and nonpregnant rats. It can be observed that higher melatonin plasma concentrations were reached in pregnant than in nonpregnant rats. Relevant pharmacokinetic parameters are listed in Table V. Pregnant rats at 19th day had much different C_{max}, AUC and Cl/F than the control group and pregnant rats at 7th day. Systemic exposure of melatonin, expressed by the mean AUC, was 41% and 206% higher for the 14th and 19th gestational day; respectively, comparing to the nonpregnant rats. Whereas the Cl/F values were 23% and 68% smaller, for the 14th, and 19th gestational day; respectively, in comparison with the control group. However, only results from the G19D were statistically different.
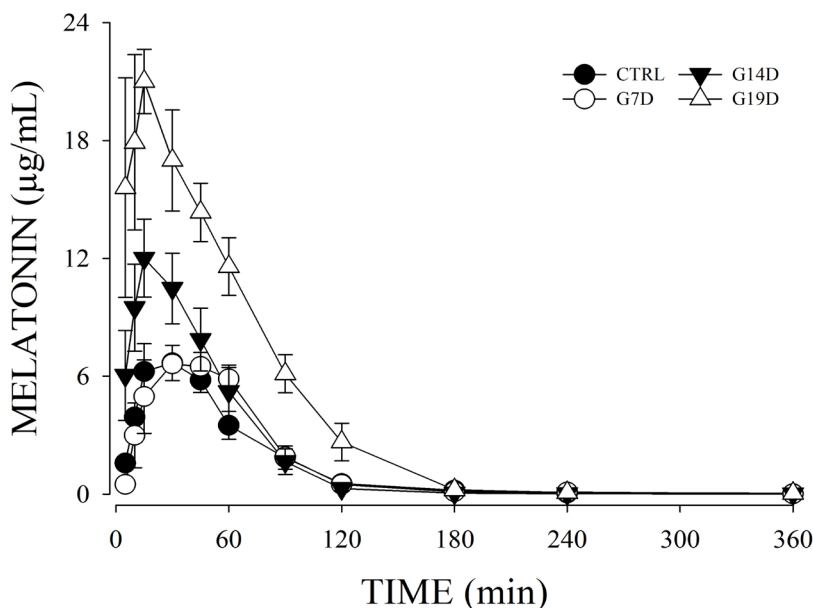

FIGURE 4 - Plasma concentration-time profiles of melatonin following administration of a single intragastric melatonin dose of 20 mg/kg in nonpregnant (CTRL) and pregnant rats on 7 (G7D), 14 (G14D) and 19 (G19D) days of gestation. Data are expressed as mean ± SEM (n=3).

TABLE V - Pharmacokinetic parameters of orally administered melatonin (20 mg/kg) in pregnant (at different gestational period) and nonpregnant rats

Parameter	Group			
	Nonpregnant	Pregnant		
		G7D	G14D	G19D
t_{max} (min)	20.0 ± 8.7	30.0 ± 15.0	15.0 ± 0.0	11.7 ± 2.9
C_{max} (µg/mL)	7.5 ± 1.6	7.5 ± 0.4	12.0 ± 3.5	22.9 ± 1.6[abc]
AUC_{last} (µg·h/mL)	450.6 ± 102.9	468.2 ± 8.1	635.2 ± 131.2	1376.7 ± 178.8[abc]
$t_{1/2}$ (min)	38.3 ± 4.4	42.3 ± 9.6	26.3 ± 6.2	33.1 ± 8.7
Cl/F (mL/min)	0.069 ± 0.017	0.064 ± 0.001	0.053 ± 0.014	0.022 ± 0.003[abc]
Vz/F (mL)	3.8 ± 0.9	3.9 ± 0.8	2.4 ± 0.4	1.08 ± 0.4[ab]

[a]Results were significantly different ($p < 0.05$) from control group. [b]Results were significantly different ($p < 0.05$) from 7th day of gestation group. [c]Results were significantly different ($p < 0.05$) from 14th day of gestation group.

t_{max}: time to reach the maximum concentration. C_{max}: maximum concentration observed. AUC: Area under the curve. $t_{1/2}$: terminal half-life. Cl/F: apparent oral clearance. Vz/F: apparent volume of distribution.

To the best of our knowledge, this study is the first to characterize gestational age-dependent changes in the oral pharmacokinetics of melatonin in a preclinical model. Our findings showed that pregnancy increases the systemic exposure of melatonin with significant difference in the last gestational stage. It is well established in both animal and human studies that melatonin exhibits extensive hepatic first pass metabolism primarily through the CYP1A2 enzyme; in fact, some authors have proposed that melatonin might be an alternative to caffeine as a probe drug for CYP1A2 phenotyping (Härtter *et al.*, 2001; 2003; Ma *et al.*, 2005). Basic and clinical investigations have evidenced a reduced expression or activity of CYP1A2 over the course of pregnancy (Tracy *et al.*, 2005; Walker, Dickmann, Isoherranen, 2011; Yu *et al.*, 2016). For example, a clinical trial included pregnant women in different gestational trimesters with the purpose to determine the CYP1A2 activity during pregnancy using salivary caffeine clearance. Enzymatic activity was significantly reduced in all trimesters comparing to the postpartum period: 32.8% in the first, 48.1% in the second, and 65.2% in third trimester (Tracy *et al.*, 2005). In an *in vitro* study, Walker, Dickmann, Isoherranen, (2011) demonstrated that CYP1A2 expression in rat hepatocytes

is decreased during pregnancy, resulting in a significantly smaller caffeine clearance (approximately 50% on the 19th day of gestation), in comparison with the control group. In addition, the authors suggest that pregnant rats can be used as a model to study mechanisms by which pregnancy decreases CYP1A2 activity in humans. Similarly, it has been demonstrated that CYP1A2 expression in mouse liver decreased on gestation days 15 and 19 compared to nonpregnant controls (Shuster *et al.*, 2013). For its part, Yu *et al.* (2016) identified a decreased metabolism of caffeine in gestational women, especially in the third trimester, emphasizing the clinical recommendations to reduce regular caffeine intake during pregnancy.

As can be observed, our results are consistent and extend the findings from these previous studies regarding the impact of pregnancy on the oral pharmacokinetics of drugs metabolized by CYP1A2 enzyme in rats and humans. Obviously, the clearance of drugs is not the only factor that determines the achieved plasma levels. The interpretations of our results must be taken with caution since pregnancy-related physiological changes can alter the absorption, distribution, metabolism and excretion of drugs, which has been widely reported (Anderson, Carr, 2009; Coppola *et al.*, 2022; Feghali,

Venkataramanan, Caritis, 2015); therefore, additional maternal physiological changes cannot be ruled out. In that sense, future investigations may consider measuring of 6-hydroxy-melatonin, the main metabolite formed from the biotransformation of melatonin through the CYP1A2 in order to determine the 6-hydroxy-melatonin/melatonin ratio.

CONCLUSIONS

We present a novel, sensitive, selective, and reproducible HPLC method with fluorescence detection for the determination of melatonin in rat plasma. This method was completely validated over a wide concentration interval (0.005 to 20 µg/mL), and it offered a good accuracy and precision. The method required only 25 µL plasma, making it very useful for studying melatonin pharmacokinetics in small animals. We have used the method successfully and demonstrated that it is an effective and inexpensive analytical alternative to determine the pharmacokinetic profile of melatonin after its exogenous administration in rats. Furthermore, in this study it was evidenced that pregnancy stage alters the oral pharmacokinetics of melatonin, increasing its plasma levels in a gestational-time dependent manner, but more markedly in the last gestational stage. Our preclinical findings may serve as additional information regarding the appropriate melatonin dosing during pregnancy.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Andersen LP, Werner MU, Rosenkilde MM, Harpsøe NG, Fuglsang H, Rosenberg J, et al. Pharmacokinetics of oral and intravenous melatonin in healthy volunteers. BMC Pharmacol Toxicol. 2016;17. https://doi:10.1186/s40360-016-0052-2.

Anderson J, Reiter R. Melatonin: roles in influenza, Covid-19, and other viral infections. Rev Med Virol. 2020;30(3). https://doi.org/10.1002/rmv.2109.

Anderson G, Carr D. Effect of pregnancy on the pharmacokinetics of antihypertensive drugs. Clin Pharmacokinet. 2009;48(3):159-168. https://doi.org/10.2165/00003088-200948030-00002.

Arreola-Espino R, Urquiza-Marín H, Ambriz-Tututi M, Araiza-Saldaña CI, Caram-Salas NL, Rocha-González HI, et al. Melatonin reduces formalin-induced nociception and tactile allodynia in diabetic rats. Eur J Pharmacol. 2007;577(1-3):203-10.

Carloni S, Proietti F, Rocchi M, Longini M, Marseglia L, D'Angelo G, et al. Melatonin pharmacokinetics following oral administration in preterm neonates. Molecules. 2017;22(12). https://doi.org/10.3390/molecules22122115.

Chen M, Stone R. Lack of effect of oral melatonin on platelet parameters in normal healthy dogs. J Am Anim Hosp Assoc. 2019;55(5):226-30.

Cheung RT, Tipoe GL, Tam S, Ma ES, Zou LY, Chan PS. Preclinical evaluation of pharmacokinetics and safety of melatonin in propylene glycol for intravenous administration. J Pineal Res. 2006;41(4):337-43.

Choudhary S, Kumar A, Saha N, Choudhary N. PK-PD based optimal dose and time for orally administered supra-pharmacological dose of melatonin to prevent radiation induced mortality in mice. Life Sci. 2019;219:31-9.

Coppola P, Kerwash E, Nooney J, Omran A, Cole S. Pharmacokinetic data in pregnancy: A review of available literature data and important considerations in collecting clinical data. Front Med. 2022;9. https://doi:10.3389/fmed.2022.940644.

Di WL, Kadva A, Johnston A, Silman R. Variable bioavailability of oral melatonin. N Engl J Med. 1997;336(14):1028-29.

Feghali M, Venkataramanan R, Caritis S. Pharmacokinetics of drugs in pregnancy. Semin Perinatol. 2015;39(7):512-19.

Gaohua L, Abduljalil K, Jamei M, Johnson TN, Rostami-Hodjegan A. A pregnancy physiologically based pharmacokinetic (p-PBPK) model for disposition of drugs metabolized by CYP1A2, CYP2D6 and CYP3A4. Br J Clin Pharmacol. 2012;74(5):873-85.

Harpsøe NG, Andersen LP, Gögenur I, Rosenberg J. Clinical pharmacokinetics of melatonin: a systematic review. Eur J Clin Pharmacol. 2015;71(8):901-9.

Härtter S, Grözinger M, Weigmann H, Röschke J, Hiemke C. Increased bioavailability of oral melatonin after fluvoxamine coadministration. Clin Pharmacol Ther. 2000;67(1):1-6.

Härtter S, Ursing C, Morita S, Tybring G, von Bahr C, Christensen M, et al. Orally given melatonin may serve as a probe drug for cytochrome P450 1A2 activity in vivo: a pilot study. Clin Pharmacol Ther. 2001;70(1):10-6.

Härtter S, Nordmark A, Rose DM, Bertilsson L, Tybring G, Laine K. Effects of caffeine intake on the pharmacokinetics of melatonin, a probe drug for CYP1A2 activity. Br J Clin Pharmacol. 2003;56(6):679-82.

Hemati K, Pourhanifeh MH, Dehdashtian E, Fatemi I, Mehrzadi S, Reiter RJ, et al. Melatonin and morphine: potential beneficial effects of co-use. Fundam Clin Pharmacol. 2021;35(1):25-39.

Hendawy A, El-Toukhey N, AbdEl-Rahman S, Ahmed H. Ameliorating effect of melatonin against nicotine induced lung and heart toxicity in rats. Environ Sci Pollut Res Int. 2021;28(27):35628-41.

Ke A, Rostami-Hodjegan A, Zhao P, Unadkat J. Pharmacometrics in pregnancy: An unmet need. Annu Rev Pharmacol Toxicol. 2014;54:53-69.

Ma X, Idle JR, Krausz KW, Gonzalez FJ. Metabolism of melatonin by human cytochromes p450. Drug Metab Dispos. 2005;33(4),489-94.

Mexican Official Norm NOM-062-ZOO-1999. Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio (2001). Official Journal of the Federation. Mexico City, Mexico. https://www.gob.mx/cms/uploads/attachment/file/203498/NOM-062-ZOO-1999_220801.pdf

Mexican Official Norm (2013) NOM-177-SSA1-2013. Que establece las pruebas y procedimientos para demostrar que un medicamento es intercambiable. Requisitos a que deben sujetarse los Terceros Autorizados que realicen las pruebas de intercambiabilidad (2013). Requirement 9.1. Official Journal of the Federation, Mexico City, Mexico. https://www.dof.gob.mx/nota_detalle.php?codigo=5314833&fecha=20/09/2013#gsc.tab=0

Miguel FM, Picada JN, da Silva JB, Schemitt EG, Colares JR, Hartmann RM, et al. Melatonin attenuates inflammation, oxidative stress, and DNA damage in mice with nonalcoholic steatohepatitis induced by a methionine- and choline-deficient diet. Inflammation. 2022;45(5):1968-84.

Moroni I, Garcia-Bennett A, Chapman J, Grunstein RR, Gordon CJ, Comas M. Pharmacokinetics of exogenous melatonin in relation to formulation, and effects on sleep: A systematic review. Sleep Med Rev. 2021;57. https://doi.org/10.1016/j.smrv.2021.101431.

NC3Rs (National Centre for the Replacement, Refinement & Reduction of Animal Research of United Kingdom). Available at: https://www.nc3rs.org.uk/ (Accessed on December 2020)

Nickkholgh A, Schneider H, Sobirey M, Venetz WP, Hinz U, Pelzl LH, et al. The use of high-dose melatonin in liver resection is safe: first clinical experience. J Pineal Res. 2011;50(4):381-88.

Roy J, Wong KY, Aquili L, Uddin MS, Heng BC, Tipoe GL, et al. Role of melatonin in Alzheimer's disease: from preclinical studies to novel melatonin-based therapies. Front Neuroendocrinol. 2022;65. https://doi.org/10.1016/j.yfrne.2022.100986.

Shah VP, Midha KK, Findlay JW, Hill HM, Hulse JD, McGilveray IJ, et al. Bioanalytical method validation--a revisit with a decade of progress. Pharm Res. 2000;17(12):1551-7.

Shuster D, Bammler T, Beyer R, Macdonald J, Tsai J, Farin F, et al. Gestational age-dependent changes in gene expression of metabolic enzymes and transporters in pregnant mice. Drug Metab Dispos. 2013;41(2):332-42.

Srinivas N. Should commonly prescribed drugs be avoided as internal standard choices in new assays for clinical samples? Bioanalysis. 2016;8(7):607-10.

Tamura H, Nakamura Y, Terrón MP, Flores LJ, Manchester LC, Tan DX, et al. Melatonin and pregnancy in the human. Reprod Toxicol. 2008;25(3):291-303.

Tracy T, Venkataramanan R, Glover D, Caritis S, National Institute for Child Health and Human Development Network of Maternal-Fetal-Medicine Unit. Temporal changes in drug metabolism (CYP1A2, CYP2D6 and CYP3A activity) during pregnancy. Am J Obstet Gynecol. 2005;192(2):633-9.

U.S. Department of Health and Human Services – Food and Drug Administration – Center for Drug Evaluation and Research (CDER) – Center for Veterinary Medicine (CVM). 2018. https://www.fda.gov/regulatory-information/search-

fda-guidance-documents/bioanalytical-methodvalidation-guidance-industry (Accessed on December 2020).

Vine T, Brown G, Frey B. Melatonin use during pregnancy and lactation: a scoping review of human studies. Braz J Psychiatry. 2022;44(3):342-8.

Walker A, Dickmann L, Isoherranen N. Pregnancy decreases rat CYP1A2 activity and expression. Drug Metab Dispos. 2011;39(1):4-7.

Wetterberg L. Melatonin and clinical application. Reprod Nutr Dev. 1999;39(3):367-82.

Xu S, Wei W, Shen Y, Hao J, Ding C. Studies on the antiinflammatory, immunoregulatory, and analgesic actions of melatonin. Drug Dev Res. 1996;39(2):167-73.

Yeleswaram K, McLaughlin L, Knipe J, Schabdach D. Pharmacokinetics and oral bioavailability of exogenous melatonin in preclinical animal models and clinical implications. J Pineal Res. 1997;22(1):45-51.

Yu T, Campbell SC, Stockman C, Tak C, Schoen K, Clark E, et al. Pregnancy-induced changes in the pharmacokinetics of caffeine and its metabolites. J Clin Pharmacol. 2016;56(5):590-6.

Zhao H, Wang Y, Yuan B, Liu S, Man S, Xu H, et al. A novel LC–MS/MS assay for the simultaneous determination of melatonin and its two major metabolites, 6-hydroxymelatonin and 6-sulfatoxymelatonin in dog plasma: application to a pharmacokinetic study. J Pharm Biomed Anal. 2016;117:390-7.

Zisapel N. New perspectives on the role of melatonin in human sleep, circadian rhythms and their regulation. Br J Pharmacol. 2018;175(16):3190-9.