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An ultra-high sensitive bioanalytical method for plasma melatonin by liquid chromatography–tandem mass spectrometry using water as calibration matrix

An-Qi Wang[a], Bo-Ping Wei[b], Yan Zhang[a], Yu-Jun Wang[a], Liang Xu[a], Ke Lan[a,*]

[a] Key Laboratory of Drug Targeting and Drug Delivery Systems, Ministry of Education, West China School of Pharmacy, Sichuan University, China
[b] Sichuan Provincial Institute for Food and Drug Control, Chengdu 610036, China

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ABSTRACT

For the endogenous substances with an ultra-low level in biological fluids, such as melatonin, the blank biological matrix is obviously not "blank". This problem leads to a serious issue of the bioanalytical methods development and validation by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). This work developed and validated an ultra-high sensitive bioanalytical method for plasma melatonin by LC–MS/MS using water as calibration matrix. The lower limit of quantitation of the method was verified to be 1.0 pg/mL and the method exhibited a linear range of 1–5000 pg/mL. Potential matrix effects, accuracy and precision were fully monitored and validated by two complementary quality control approaches respectively using water and the pooled plasma as matrix. The intra-run and inter-run precisions were less than 11.5% and 12.2%, respectively, and the relative error was below ±13.8% for all of 5 quality control levels. The method was successfully applied to investigate the daytime (8:00 AM–8:00 PM) baseline level of endogenous plasma melatonin, as well as the pharmacokinetic profiles of exogenous melatonin after oral administration in beagle dogs.

1. Introduction

Melatonin, the principal hormone of the pineal gland [1], plays an important role in regulating the circadian rhythms of several biological functions, such as sleep, mood, and perhaps reproduction, tumor growth, and aging [2]. Circulating level of endogenous melatonin is highly variable individually [2,3], low around 10 pg/mL in the daytime and high around 25–120 pg/mL at night [4]. An ultra-high sensitive bioanalytical method is demanded to quantify melatonin in biological fluids.

Current bioanalytical methods available for the determination of melatonin in biological fluids include immunoassay (IA), such as enzyme immunoassay (EIA) [5] and radioimmunoassay (RIA) [6], GC–MS [7–10], high-performance liquid chromatography (HPLC) with fluorescence detection [11–15] or electrochemical detection [16–18], as well as liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) [19]. The common advantages and pitfalls of IA, GC–MS, HPLC and LC–MS/MS had been well discussed previously [20]. Even though RIA has potential cross reactivity, it prevails in the most recent reports that monitored melatonin level in biological fluids [21–23], due to its ultra-high sensitivity, with a lower limit of quantitation (LLOQ) less than 1 pg/mL. Compared to RIA, LC–MS/MS demonstrates less sensitivity, and is not widely applied to the studies of melatonin. To date, the publications on the bioanalytical method development and validation of melatonin by LC–MS/MS are still limited [19,20,24–28]. With the technical advances of the LC–MS instruments, LLOQ of melatonin in biological fluid had been improved from 4 ng/mL [24] to 2 pg/mL [20].

In the field of bioanalytical development and validation, a basic principle is that the same biological matrix as the matrix in the intended samples should be used to validate the selectivity and the involvements of matrix effects. Thus, a serious issue emerged while using LC–MS/MS to quantify endogenous substances with an ultra-low level in biological fluids, such as melatonin. That is said, with the improvement of LLOQ, the blank biological matrix may obviously become not "blank" and may not be used for calibrations. For the instance of melatonin, two reports, which determined endogenous melatonin in human saliva, dodged this problem by setting a criterion that the daytime saliva absence of melatonin (<20% of LLOQ) was used as blank matrix [20,26]. The most recent report, which quantified melatonin in human plasma, deal with this problem by using a dichloromethane extracted "clean" plasma as the blank matrix [27]. In a similar way, the simplest strategy is to use fresh water as the blank matrix to prepare the calibrations. However, the major technical challenge is that the potential matrix effects should be carefully assessed and monitored if a different matrix was used. Therefore, the purpose of this work is to develop and validate an ultra-high sensitive bioanalytical method of plasma melatonin by LC–MS/MS using water as calibration matrix.

* Corresponding author. Tel.: +86 028 85503722; fax: +86 028 85503722.
E-mail addresses: lanwoco@scu.edu.cn, lanwoco@163.com (K. Lan).

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2. Experimental

2.1. Chemicals and reagents

Melatonin (99.9%) was purchased from the USP (Rockville, MD). N-acetyltryptamine used as internal standard (IS) was obtained from Sigma (St Louis, MO). Molecular structures of melatonin and IS were shown in Fig. 1. Acetonitrile (Fisher), methanol (Fisher), formic acid (Dikma), ammonium formate (Fluka), ammonium hydroxide solution (Fluka, 10% in water), dichloromethane (TEDIA, pesticide level) were used as received. Water was prepared by arium® pro Ultrapure Water Systems (Sartorius Stedium, Göttingen, Germany).

2.2. Animals and plasma samples

2.2.1. Pooled plasma

6 Beagle dogs (3 male and 3 female, age about 6 months, weight 7.1 ± 0.7 kg) were obtained from the Center of Experimental Animals, Sichuan University. Each dog was housed in one cage in temperature-controlled rooms, with a dark period from 8:00 PM to 8:00 AM, and was allowed for the standard laboratory food and water for 1 month. At this period, daytime venous blood was collected from each dog, and the heparin-anticoagulated plasma was separated and pooled in batches for the use of method development and validation.

2.2.2. Pharmacokinetics

In a 3-way crossover study, six dogs received per oral administration of 2 mg Circadin® (melatonin prolonged-release tablets, RAD Neurim Pharmaceuticals EEC Ltd., 2 mg/tablet, Lot: 457105801), 2 mg melatonin (encapsuled in lab) and 1 vehicle gelatin capsule in the three continuous experimental periods, with a 1-week washout between periods. In each period, dogs were administrated with Circadin®, melatonin or vehicle at 8:00 AM according to the standard 3-way crossover study design. 45 min before oral administrations, a standard diet was given, and sequentially the venous blood was collected in heparinized vacuum tubes. After that, venous blood were collected post drug administration at 8:10, 8:20, 8:40, 9:00, 9:30, 10:00, 11:00, 12:00, 14:00, 16:00, 18:00 and 20:00. Blood samples were centrifuged at $1500 \times g$ for 10 min, and plasma was separated and frozen at $-20\,^{\circ}C$ until analysis. The animal experiments were conducted in compliance with the standard animal use practices and were approved by Institutional Committee on Animal Uses at Sichuan University.

2.3. Calibration and quality control samples

Stock solutions (1.00 mg/mL) of melatonin and IS were made up in acetonitrile and stored at $-20\,^{\circ}C$ for a maximum period of 2 weeks. On the day of analysis, a dilution of the IS stock solution was prepared in water to give a working concentration of about 20 ng/mL. Working standard solutions of melatonin (50,000, 10,000, 5000, 1000, 500, 100, 50, 20, 10 pg/mL) were prepared by serial dilution of the stock solutions with water. Quality control (QC) standard solutions (50,000, 5000, 500, 100 and 10 pg/mL) were prepared in a similar manner.

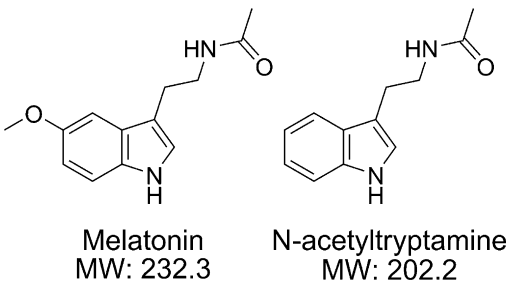

Fig. 1. Molecular structure of melatonin and the internal standard, N-acetyltryptamine.

Due to the occurrence of endogenous melatonin in the pooled plasma, 50 μL melatonin working standard solutions were spiked into 500 μL water instead of the pooled plasma to prepare the calibration curve, giving the calibration concentrations of 5000, 1000, 500, 100, 50, 10, 5, 2, 1 pg/mL. Single batch of pooled plasma was thawed at room temperature. Two complementary groups of QC samples were prepared on the day of analysis in six replicates. The first group of QC samples was prepared by spiking 50 μL working standard solutions (100 and 10 pg/mL) into 500 μL water. The second group of QC samples was prepared by spiking 50 μL working standard solutions (50,000, 5000 and 500 pg/mL) into 500 μL pooled plasma, whose basal concentration was simultaneously determined in triplicates.

2.4. Sample extraction

Glass tubes and glass autosampler vial inserts, which were heat-treated at $300\,^{\circ}C$ for at least 2 h (Isotemp Programmable Forced-Draft Furnace, Fisher Scientific), were used for sample preparations. Calibration samples, QC samples and unknown samples were ready for extraction in the heat-treated glass tubes with a total volume of 550 μL. 50 μL of N-acetyltryptamine working solution was added and mixed well. Subsequently, 50 μL of ammonium hydroxide solution (10% in water) was added to alkalify the plasma. After being briefly shaken, samples were extracted with 2 mL of dichloromethane following vortex mixing at 600 rpm for 5 min (Vortex Genie-2, Scientific industry, US). The tube was centrifuged for 10 min at 3000 *g* at $4\,^{\circ}C$ (Eppendorf Centrifuge 5810R, Hambury, Germany), and the lower organic phase was transferred to another heat-treated glass tube with the procedures described below. Reach the pipette tip through the aqueous layer to the organic layer. Slightly release several air bubbles to deplete the aqueous solution in the tip. Draw the organic phase as much as possible. The organic phase was evaporated to dryness under a stream of nitrogen at room temperature (TurboVap® LV, Caliper life science, USA). The residue was reconstituted in 100 μL of water by vortex mixing at 600 rpm for 2 min. After being centrifuged for 5 min at 3000 *g*, the supernatant was transferred into the heat-treated glass autosampler vial inserts.

2.5. Chromatographic and mass spectrometric conditions

HPLC analyses were performed on an Agilent Technologies 1200 series system equipped with a G1322A degasser, a G1312B SL binary pump, a G1316B SL thermostated column compartment, and a HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). An Agilent ZORBAX Eclipse XDB C18 column (4.6 mm i.d. × 150 mm, 5 μm) was maintained at $20\,^{\circ}C$ equipped with a Phenomenex Security-Guard C18 guard column (4 mm × 3.0 mm i.d). The injection volume was 10 μL and the injector needle was washed with 100 μL of both methanol–water (20:80) and methanol–water (80:20), once before injection and for 5 times after injection. The aliquots of samples were gradient-eluted at 0.5 mL/min using (A) 2 mM ammonium formate and 0.1% formic acid in water and (B) acetonitrile (60% A for 1 min, 60–25% A in 4 min, 25–60% A in 3 min, post run 3 min) with a total run time of 11 min.

An Agilent 6460 triple-quadrupole mass spectrometer was operated with an Agilent G1948B ionization source in positive ESI mode. An Agilent MassHunter workstation was used for the control of equipment, data acquisition, and analysis. The MS/MS

parameters of melatonin and N-acetyltryptamine were automatically optimized by the Optimizer of MassHunter workstation. After that, the instrument was operated with the capillary voltage at +4.0 kV, and nozzle voltage at +500 V. Nitrogen was used as a nebulizer gas of 20 psi, a carrier gas of 11 L/min at 350 °C, and a sheath gas of 7 L/min at 250 °C. Multiple reaction monitoring (MRM) was employed for data acquisition. The optimized MRM fragmentation trasitions were m/z 233.1 → m/z 174, with a fragmenter voltage of 110 V and collision energy (CE) of 9 V for melatonin, m/z 203.1 → m/z 144 with a fragmenter voltage of 80 V and CE of 9 V for N-acetyltryptamine.

2.6. Determination of selectivity, linearity, carry over, matrix effects, accuracy and precision

The selectivity was investigated by assaying the blank, which used water as matrix and was prepared in accordance with the sample extraction procedure. Linearity was assessed by plotting calibration curves prepared with water as matrix. Carry over was analysed by assay the blank injected after the analysis of the highest QC sample (5000 pg/mL). Matrix effects, accuracy and precision were assessed by two complementary groups of QC samples described in Section 2.3. Intra-run precision and accuracy were assessed by measuring 6 samples in one batch and inter-run precision and accuracy were assessed by measuring those 6 samples on three different days. The accuracy was expressed as the RE (relative error), which was calculated as (observed concentration – added concentration)/(added concentration) × 100% for the first group of QC samples, and (observed concentration – pooled plasma's basal concentration – added concentration)/(added concentration) × 100% for the second group of QC samples. The accuracy was required to be within ±15%, and the intra- and inter-day precisions were not to exceed 15%.

2.7. Stability

The stability of melatonin in the pooled plasma was evaluated by analysing replicates (n = 3) of the second group of QC samples that had been exposed to different conditions (at room temperature for 12 h, at −20 °C for 2 weeks, at the sampler plate for 24 h, after three freeze–thaw cycles). The analytes were considered stable in the biological matrix when the RE was within ±15%.

3. Results and discussions

3.1. Analytical conditions

LC–MS/MS conditions were optimized based on the report of Yang et al. [25]. Table 1 presented the precursor ion, the product ions and the MS–MS parameters of both melatonin and N-acetyltryptamine optimized by Optimizer of Agilent MassHunter workstation. The transients with the highest abundance were selected for MRM detection (m/z 233.1 → 174 for melatonin, m/z 203.1 → 144 for N-acetyltryptamine). The results were consistent with the previous reports.

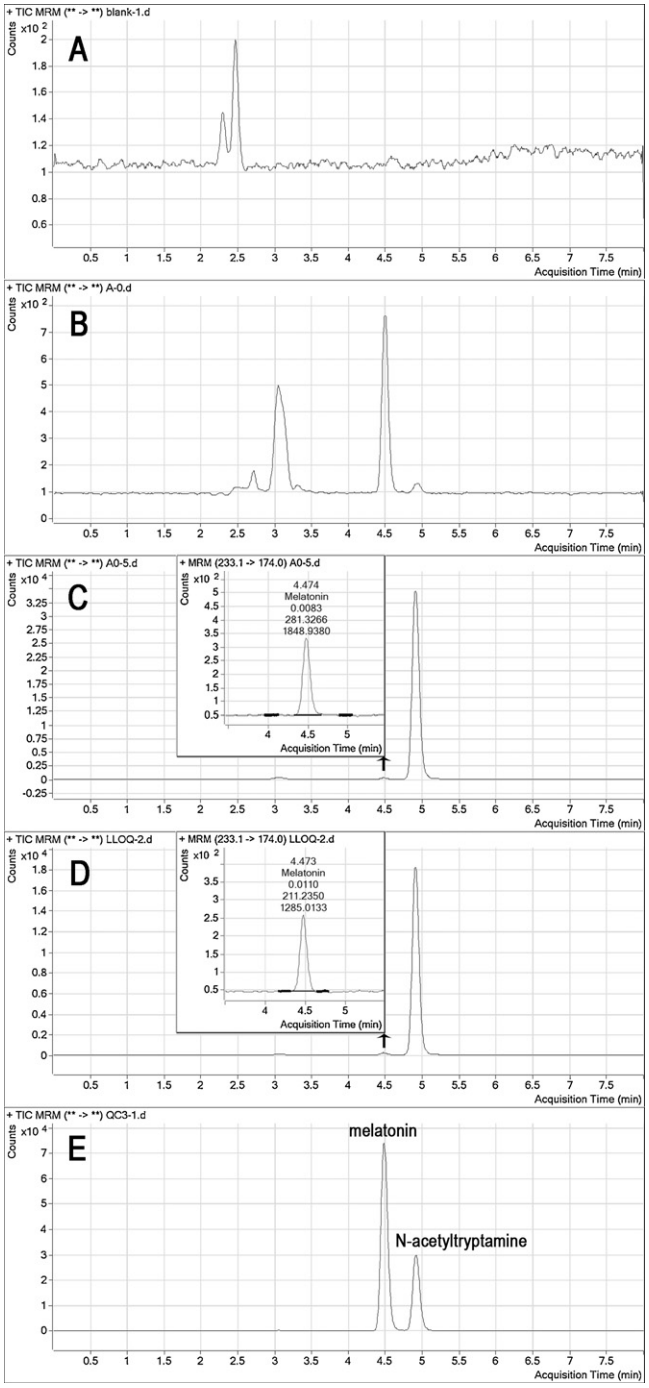

Fig. 2. Typical MRM total ion chromatograms of (A) the blank, which used water as matrix and was prepared according to the sample extraction procedure; (B) The pooled plasma without addition of IS (N-acetyltryptamine). Signal of IS illustrated a typical response of carry over; (C) The pooled plasma with addition of IS; (D) LLOQ (1.0 pg/mL); (E) The highest quality control (about 5000 pg/mL).

Table 1
The ESI positive precursor ion and the product ions of melatonin and N-acetyltryptamine.

Chemical	Melatonin				N-acetyltryptamine			
Precursor ion (amu)	233.13	233.13	233.13	233.13	203.12	203.12	203.12	203.12
Fragmentor (V)	110	110	110	110	80	80	80	80
Product ion (amu)	174	159	130.4	143	144	117	175.6	83.8
Collision energy (V)	9	25	40	29	9	33	13	25
Abundance	23,880	6459	1150	2383	10,809	1519	48	50

Table 2
Intra-run and inter-run assay precision and accuracy for melatonin in beagle dogs plasma.

QC level	Run	Concentration (pg/mL)			RSD (%)		RE (%)
		Basal ($n=3$)	Added	Observed ($n=6$)	Intra-run	Inter-run	
1[#]	1	0.00	1.00	1.12 ± 0.12	11.0	12.2	12.0
	2	0.00	1.00	1.14 ± 0.11	9.6		13.8
	3	0.00	1.01	0.95 ± 0.07	7.4		−5.4
2[#]	1	0.00	10.03	11.25 ± 0.94	8.4	11.2	12.1
	2	0.00	10.03	10.95 ± 1.26	11.5		9.2
	3	0.00	10.08	10.86 ± 1.00	9.2		7.7
3[*]	1	58.1 ± 4.5	50.2	112.3 ± 2.8	2.5	5.6	8.1
	2	40.7 ± 1.8	50.2	91.2 ± 2.6	2.9		0.6
	3	17.8 ± 1.6	50.4	72.0 ± 1.5	2.2		7.5
4[*]	1	58.1 ± 4.5	501.5	606.7 ± 23.8	3.9	3.8	9.4
	2	40.7 ± 1.8	501.5	550.3 ± 19.8	3.6		1.6
	3	17.8 ± 1.6	504.0	557.2 ± 12.2	2.2		7.0
5[*]	1	58.1 ± 4.5	5015.0	5326.5 ± 105.8	2.0	4.4	5.1
	2	40.7 ± 1.8	5015.0	5528.3 ± 132.0	2.4		9.4
	3	17.8 ± 1.6	5040.0	5718.3 ± 75.5	1.3		13.1

The accuracy was expressed as the relative error (RE).
[#] The RE was calculated as (observed concentration − added concentration)/(added concentration) − 100%.
[*] The RE was calculated as (observed concentration − basal concentration − added concentration)/(added concentration) × 100%.

3.2. Selectivity, linearity, limit of quantification and carry over

In this study, the selectivity was investigated by comparatively assaying the real blank sample extracted from water and the extracted plasma sample. Typical ion chromatograms of the blank, the pooled plasma with and without addition of IS, LLOQ and highest QC were shown in Fig. 2. It was clear that endogenous melatonin in the pooled plasma compromised the calibration of melatonin. Such a strategy for selectivity testing had taken for granted that the employed MRM transient (m/z 233.1 → 174) is unique for melatonin at the intended retention time. However, this strategy may fail, if some substances in the biological samples exhibit the same MRM transient and a similar chromatographic performance as the analyte. For further validation of the selectivity of such kind of bioanalytical methods, it is recommended that the relative intensities of different MRM channels be monitored and compared between the extracted plasma sample and the neat standard.

Water was used as matrix to prepare the calibration curves. The method exhibited a linear range of 1–5000 pg/mL. The calibration curve fitted to a $1/c$ weighted linear regression. The mean equation (mean ± SD of curve coefficients) of the calibration curves ($n=9$) obtained throughout the method validation and applications was $y = 0.4713\text{E-}3\ (\pm 0.0554\text{E-}3)x + 0.0053(\pm 0.0037)$ (correlation coefficient $r = 0.9981 \pm 0.011$), where y represents the melatonin peak area to IS peak area ratio and x represents the corresponding nominal melatonin concentration (pg/mL).

The LLOQ of the method was verified as 1.0 pg/mL. The signal to noise ratio (S/N) of LLOQ was more than 30, depending on the transferring percentage of organic phase after samples extraction. The sensitivity of the present method was much higher than previous LC–MS/MS methods, and equivalent to the commercial RIA method. Because of the ultra-high sensitivity of the method, melatonin and IS residues in the apparatus caused problems in method development. In this work, the washing procedure of the injector needle was optimized with two solvents with distinct polarities. The sample extraction apparatuses may be reused only after being heat-treated at 300 °C for at least 2 h to remove the residues. In these ways, the S/N of carry over for melatonin or IS was less than 5. A typical carry over of IS was illustrated in Fig. 2B.

3.3. Matrix effect, precision and accuracy

The matrix effects are derived from the potential ion suppression or enhancement owing to the co-eluting matrix components in the biological samples. In this work, the second group of QC samples was designed to assess the matrix effects. Matrix effects did not

Table 3
Stability of melatonin in beagle dogs plasma.

QC level	QC level	Concentration (pg/mL)			RSD (%)	RE (%)
		Basal	Add	Found ($n=3$)		
Freeze–thaw (three cycles)	3	58.1	50.2	103.9 ± 7.1	6.8	−8.8
	4	58.1	501.5	498.8 ± 24.0	4.8	−12.1
	5	58.1	5015.0	4974.3 ± 231.0	4.6	−2.0
Autosampler, 24 h	3	58.1	50.2	111.8 ± 5.0	4.5	7.1
	4	58.1	501.5	595.4 ± 52.1	8.7	7.1
	5	58.1	5015.0	5442.3 ± 108.4	2.0	7.4
Ambient, 12 h	3	58.1	50.2	115.8 ± 8.0	6.9	14.9
	4	58.1	501.5	602.1 ± 21.1	3.5	8.5
	5	58.1	5015.0	5386.8 ± 95.7	1.8	6.3
Freezing, 2 weeks	3	58.1	50.2	102.9 ± 9.2	8.8	−10.9
	4	58.1	501.5	561.1 ± 19.3	3.4	0.3
	5	58.1	5015.0	5526.5 ± 84.7	1.5	9.0

The accuracy was expressed as the relative error (RE), The RE was calculated as (observed concentration − basal concentration − added concentration)/(added concentration) × 100%.

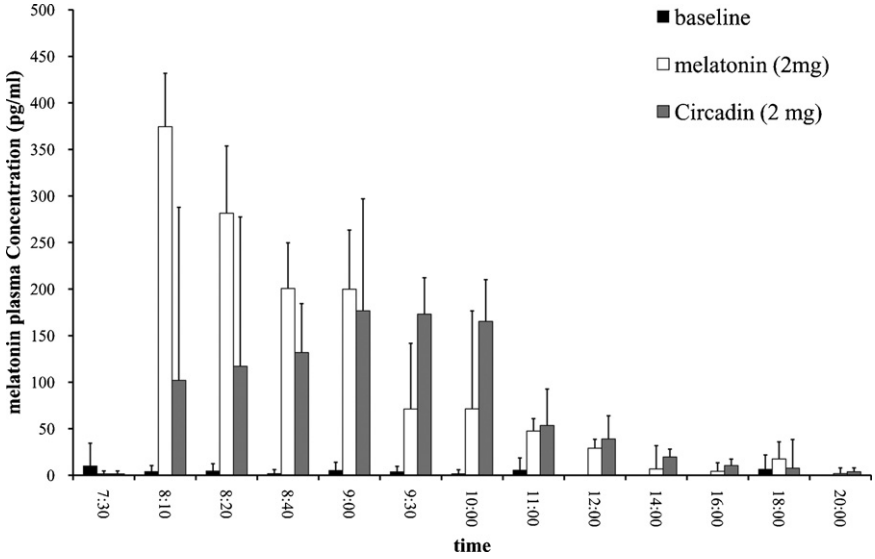

Fig. 3. Daytime baseline of the endogenous plasma melatonin, as well as the exogenous plasma melatonin level after oral administration of 2 mg of Circadin® (melatonin prolonged-release tablets) or 2 mg of melatonin in gel capsules in beagle dogs ($n = 6$).

occur in the calibration samples prepared with water, and might occur in the samples prepared with the pooled plasma. The influences of the matrix effects on the performance of the method were indirectly assessed using QC samples prepared in pooled plasma. The average calculated concentration of 50, 500, and 5000 pg/mL melatonin added in the pooled plasma were within 85–115% of their nominal concentrations, indicating that matrix effects did not affect the accuracy of the method. In addition, different batches of pooled plasma were used to prepare QC samples. These QC results also performed well. Hence, it is supposed that with the technical advances of separation sciences, the matrix effects might have a more and more minor effect on the performances of LC–MS/MS methods.

The precision and accuracy were calculated based on six replicate analyses of the two complementary groups of QC samples (total 5 concentration levels) at three analytical runs. The accuracy and precision data are shown in Table 2. In this study, the intra-run and inter-run precisions were less than 11.5% and 12.2%, and the RE was less than ±13.8% for each QC level. These data indicated that the method was reproducible, accurate and reliable.

3.4. Extraction recovery and stability

The recoveries of melatonin extracted from biological fluid by dichloromethane had been reported to be more than 90% [13]. The accuracy and precision of the present method verified this report. The stability of melatonin in biological samples had also been fully evaluated previously. In the present study, the stability of melatonin in plasma was studied throughout the critical steps. The results are shown in Table 3, demonstrating that melatonin was stable in plasma for 24 h at room temperature, for 2 weeks at −20 °C, after three freeze–thaw cycles, and in water at room temperature for 24 h after being extracted and reconstituted.

3.5. Application of the method

This bioanalytical method was successfully applied to investigate the daytime (8:00 AM–8:00 PM) baseline level of endogenous plasma melatonin, as well as the pharmacokinetic profiles of exogenous melatonin after oral administration of 2 mg of Circadin® or melatonin in beagle dogs. Results were shown in Fig. 3. These results clearly demonstrated the applicability of the present method in the study of the function of pineal gland as well as the pharmacokinetics of low dose of melatonin.

4. Conclusion

This work developed and validated an ultra-high sensitive bioanalytical method for plasma melatonin by liquid chromatography–tandem mass spectrometry using water as calibration matrix. The potential matrix effects, accuracy and precision were fully monitored and validated by two complementary quality control approaches, in which water was used as matrix to assess the accuracy and precision of the low concentration (1 pg/mL and 10 pg/mL), the pooled plasma was used as matrix to assess the matrix effects, accuracy and precision of the high concentration (50, 500 and 5000 pg/mL). The method was successfully applied to the determination of the endogenous and exogenous melatonin in beagle dogs' plasma. From the results of all the validation results and applicability of the method, it was concluded that the present method was sensitive, fast and robust for the determination of plasma melatonin in beagle dogs.

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