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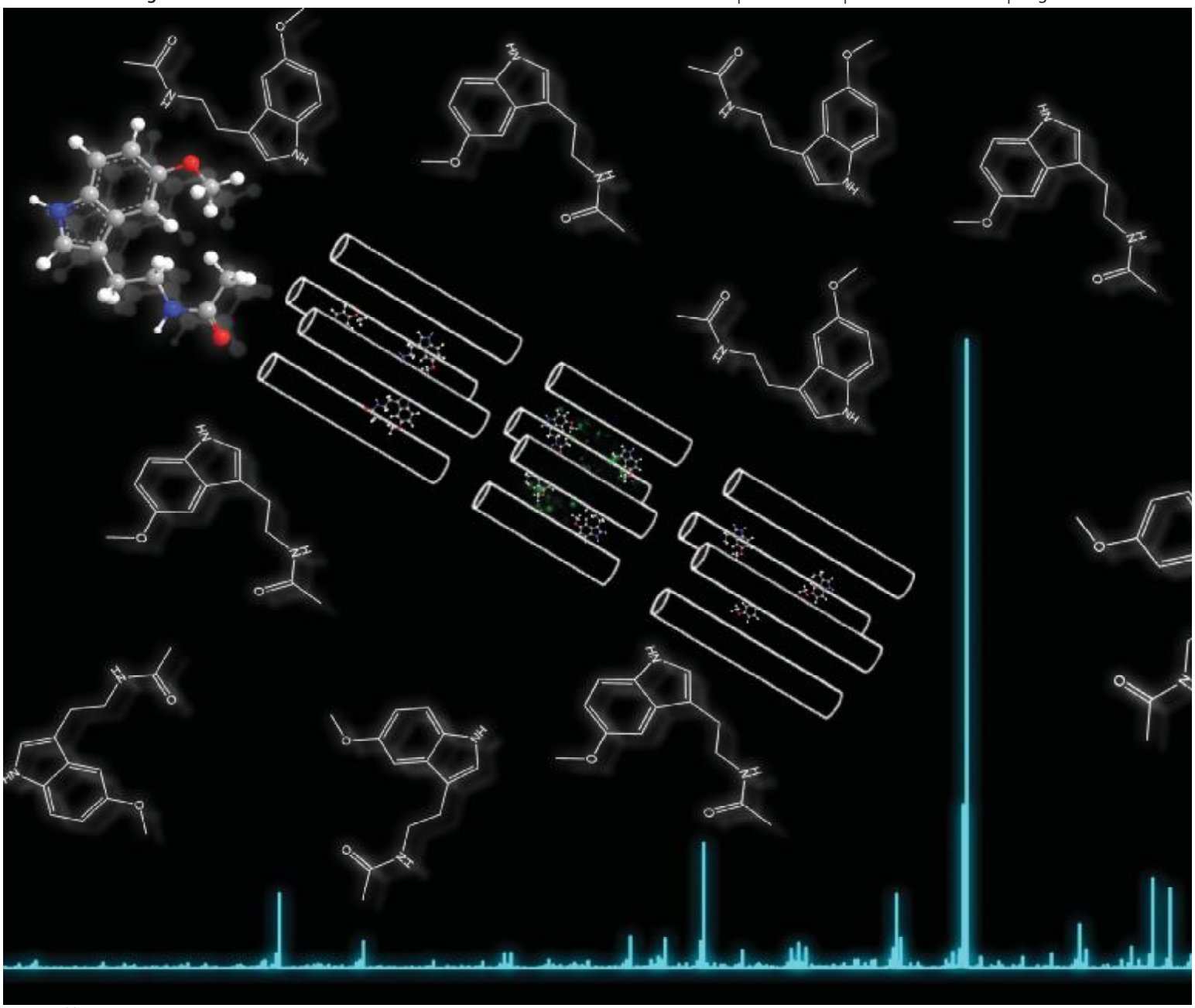

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Rosineide C. Simas *et al.*

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Assessing melatonin and its oxidative metabolites amounts in biological fluid and culture medium by liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS)

Mirela B. Coelho,[a] Maria Carolina Rodrigues-Cunha,[b] Christina R. Ferreira,[a] Elaine C. Cabral,[a] Guilherme P. Nogueira,[c] Marcos N. Eberlin,[a] Claudia L. V. Leal[b] and Rosineide C. Simas*[ac]

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The introduction of precise analytical technologies in the biological area is needed to increase the knowledge of the fundamental processes occurring in the animal and human domain. The objective of this study was to develop a highly sensitive and selective method based on liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) using electrospray ionization for the simultaneous detection and quantification of melatonin (MEL) and its metabolites, 6-hydroxymelatonin (6-HMEL), N^2-acetyl-N^1-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-metoxykynuramine (AMK). Using this methodology, we have studied the role of melatonin and its metabolites in the mammalian embryo *in vitro* production system, which is fundamental due to the antioxidant and signaling roles of these compounds. Samples comprised of bovine follicular fluid (FF) and tissue culture medium (TCM 199). The optimized procedure uses liquid–liquid extraction with MS monitoring *via* selective reaction monitoring (SRM). Low limits of detection/quantification were obtained, 3/10 pg mL^{-1} for MEL, AFMK and AMK and 30/100 pg mL^{-1} for 6-HMEL, respectively using deuterated melatonin (MEL-d_4) as the internal standard (IS). Validation and correlation coefficients were higher than 0.999 and recoveries were 80–108%. Precision was evaluated as repeatability and intermediate precision with relative standard deviation values <3.55%. The method has been successfully applied to the analysis of pooled FF and TCM 199 samples and results suggest that MEL and its metabolites are involved in oocyte maturation and that their proper quantitation is essential to monitor and study their role in such a process.

Introduction

Melatonin (N^1-acetyl-5-metoxytryptamine) was discovered in 1958 by Lemer *et al.*[1] It is an indolamine molecule derived from tryptophan and is endogenously produced in all organisms such as bacteria,[2] plants,[3] invertebrates[4] and non-mammalian[5] and mammalian vertebrates.[6] It mediates diverse physiological actions due to its immunomodulatory and cytoprotective properties.[7,8] Also, seasonal changes in melatonin production are responsible for seasonal breeding of various animal species. In humans, melatonin is believed to have lower impact on reproduction but to play an essential role in sleep and wakefulness, known as the circadian cycle.[9]

In mammals, melatonin is produced by the pineal gland and is enzymatically or non-enzymatically metabolized in all cells. The main metabolites of melatonin are 6-hydroxymelatonin (6-HMEL), which is produced by cytochrome P-450; N^2-acetyl-N^1-formyl-5-methoxykynuramine (AFMK), which is produced by several enzymatic and non-enzymatic mechanisms; and N^1-acetyl-5-metoxykynuramine (AMK) which is produced by deformylation of AFMK.

In the reproductive system, free radicals are generated by the follicular metabolism in ovaries and all melatonin metabolites are scavengers of free radicals.[10–12] Free radicals are also present in the microenvironment around an oocyte, which is the follicular fluid (FF). Changes in the FF microenvironment influence follicular development, ovulation, oocyte quality, sperm–oocyte interaction, implantation and embryo development.[13]

Classical methods for the determination of melatonin in biological samples have mainly used fluorimetry bioassays. Today, new technologies with high performance and high

[a]*ThoMSon Mass Spectrometry Laboratory – Institute of Chemistry, University of Campinas (UNICAMP), Campinas, SP, Brazil. E-mail: simas.rc@gmail.com; Fax: +55 19 3521 3073; Tel: +55 19 3521 3049*

[b]*Faculty of Animal Sciences and Food Engineering – FZEA, University of São Paulo – USP, Pirassununga, SP, Brazil*

[c]*DAPSA, Faculty of Veterinary Medicine, São Paulo State University-UNESP, Araçatuba, SP, Brazil*

sensitivity, such as high pressure liquid chromatography (HPLC) with reverse phase and fluorometric or electrochemical detection, and gas chromatography (GC) coupled to MS, have been applied and reported for sample quantitation.[14] Derivatization is sometimes employed to generate fluorophores, but the procedure displays low specificity, since other components of the biological sample can also bind to fluorophores, interfering with specific quantification of melatonin. Melatonin monitoring in biological fluids by immunological methods has also been widely employed with low levels of detection, but cross-reactions may be a limitation.[15,16]

Perhaps the most widely used method for melatonin determination in biological fluids, such as in FFs and *in vitro* oocyte maturation studies, is currently the radioimmunoassay.[17–19] This method is based on antigen–antibody reaction and utilizes antibodies conjugated with radioisotopes, which may be based on I^{125} or I^{131}, cobalt or tritium. Low levels of quantitation are achieved, but immunoassays present considerable drawbacks such as short half-life of reagents, operational risk and the need for special training and cautions and costly discharge of material due to biosecurity.[20] GC–MS is sensitive and provides greater specificity, but the need for derivatization makes it time-consuming. LC–MS/MS is, therefore, a valuable alternative that avoids the various drawbacks of other techniques, providing high sensitivity and simple sample preparation protocols.

Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has been widely used in the field of animal production, such as for the analysis of dairy products, for the detection and quantitation of veterinary drugs,[21–24] and for the evaluation of epigenetic alterations in cloned animals.[25,26] Applications of LC–MS/MS in the area of animal reproduction are, however, broader and new applications are increasingly reported. Therefore LC–MS/MS represents an analytical technique suitable for the analysis of melatonin and its metabolites in biological fluids.

Besides the determination of selected compounds by LC–MS/MS, the selectivity and broad monitoring abilities of this technique also allows one to investigate the presence of molecules with similar or the same chemical structure or class. The use of the multiple reaction monitoring (MRM) mode allows quite low detection limits, due to the increased signal/noise ratio and high selectivity.

For quantitative purposes, one must also consider the matrix effect, which may result in suppression of the analytical signal due to competition from other compounds present in the sample during ionization. To eliminate or minimize matrix effects, proper chromatographic separations coupled with selective extraction procedures are normally applied. Among the sample preparation techniques employed in bioanalytical methods, liquid–liquid extraction (LLE) and solid phase extraction (SPE) are the most commonly used approaches.[27]

The main objective of this work was to develop, validate and apply an HPLC–ESI-MS/MS method for the determination of melatonin and its metabolites. The method was tested in the analysis of ovarian follicular fluid (FF)[28] and tissue culture medium (TCM 199) used for oocyte *in vitro* maturation of bovine species, which was selected as a biological model.

Material and methods

Chemicals and materials

Unless otherwise stated, reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, ethyl acetate, tert-butyl methyl ether (TBME) and acetone were of liquid chromatographic grade. Ammonium hydroxide and formic acid were of analytical grade (≥98% purity). Ultrapure water (Millipore, Bedford, MA, USA) was used for all aqueous solution preparation. The standards used were melatonin (MEL analytical grade ≥98% purity); melatonin-d_4 (MEL-d_4, analytical grade >98% purity; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA); 6-hydroxymelatonin (6-HMEL, analytical grade ≥98% purity); N^1-acetyl-N^2-formyl-5-metoxykinuramine (AFMK, analytical grade >98% purity) and N^1-acetyl-5-methoxykynuramine (AMK, analytical grade >98% purity) which were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz Biotechnology, Inco. Santa Cruz, CA, USA).

Stock solutions were prepared in methanol at a concentration of 100 μg mL^{-1} and working solutions were made from stock solutions by dilution with ultrapure water to reach a concentration of 150 ng mL^{-1}. All the solutions were stored in dark glass at −20 °C before analysis, stock solutions were used for up to 6 months and working solutions for up to 2 weeks.

Spiked and calibration standards at various concentrations were prepared by combining aliquots of working solutions and internal standards (IS) with the LC mobile phase. These solutions were stored in amber glass at −20 °C for up to 2 days. Calibration standards mixtures had the following final concentrations: 12.5, 50, 150, 250, and 750 pg mL^{-1} for AMK and AFMK; 15, 100, 300, 500, 1000, and 1500 pg mL^{-1} for MEL; 100, 150, 500, 1000, and1500 and 2.5, 5.5, 7.5, 12.5, and 25 ng mL^{-1} for 6-HMEL. These solutions were prepared daily from the stock solutions by serial dilutions. The concentration of the IS in all the calibration standards mixtures and final sample solutions was 1000 pg mL^{-1}. Tuning solutions (0.5 μg mL^{-1}) were freshly prepared in methanol containing 0.1% formic acid.

Instrumentation

For sample preparation, a concentrator Eppendorff 5305 plus (Hamburg, Germany), Eppendorf Centrifuge 5418 (Rotor FA-45-18-11, Hamburg, Germany) and mixer IKA® Vibrax VXR Basic (IKA Works Inc., Wilmington, NC) were utilized. For quantitation, an HPLC system (Agilent Technologies 1260 series, Waldbronn, Germany) coupled to a Q-TRAP 5500 tandem mass spectrometer (AB Sciex, Concord, ON, CA) equipped with an electrospray source were used. Data acquisition and processing was performed with Analyst 1.6.1 and Multiquant 1.2 software packages.

Chromatographic conditions

Chromatographic separation of analytes was performed on a Kinetex C-18 reversed phase column (50 mm × 3.0 mm i.d., 2.6 μm particle size, Phenomenex, Torrance, CA) with a compatible pre-column (C-18, PN 00D-4462-YO, Phenomenex, Torrance, CA) at a flow rate of 0.52 mL min^{-1}. The analysis time

was 3.5 min and the injection volume was 10 μL. The autosampler was kept at 10 °C, the column at 30 °C and acetone was used for a 45 s needle wash between samples. The isocratic mobile phase was composed of acetonitrile/water (80 : 20, v/v).

MS/MS operating conditions

The electrospray source was operated in the positive ion mode (ESI+). Nitrogen was used as the curtain (10 psi), nebulizer (45 psi), auxiliary (20 psi) and collision (high or 12 a.u.) gas. The ion transfer voltage was set to 4500 V, and the probe temperature to 500 °C. Sample analysis was carried out in the MRM mode with a dwell time of 50 ms per channel. Compounds and source-dependent parameter optimizations were performed by infusion of standard solutions. The most sensitive fragment signal was selected as the quantification ion pair, while the second highest fragment signal was selected as the confirmation ion pair as shown in Table 1. All other conditions were automatically optimized by the instrument during sample injection.

Samples

Tissue culture medium 199 (TCM 199, Sigma-Aldrich Chemical Inc, St Louis, MO, USA) and bovine FF were used for quantification of melatonin and its metabolites. Medium samples of *in vitro* culture were collected at two different times, 0 and 24 h. These evaluations were aimed at determining the behavior of melatonin and its metabolites in physiological conditions (FF) and under *in vitro* culture conditions normally used for bovine oocyte *in vitro* maturation (TCM 199). TCM 199 was prepared as for a normal defined maturation culture (TCM 199 was supplemented with 0.1% polyvinyl alcohol, 0.25 mM sodium pyruvate and 100 IU mL^{-1} penicillin). Droplets of medium (100 μL) were placed in sterile plastic 35 mm Petri dishes and covered with mineral oil. Dishes were cultured for 24 h under 5% CO_2 in air and maximum humidity at 38.5 °C. Medium samples were collected at the end of culture. Freshly prepared medium (0 h) samples were also collected. Regarding FF, the samples were recovered from cow ovaries collected at a local slaughterhouse by aspiration of follicles with a needle (18G) coupled to a 10 mL disposable plastic syringe. Follicles were aspirated and the FF was placed into 50 mL plastic tubes, separately, according to follicle diameter (small follicles <6 mm and large follicles >8 mm, measured with a caliper). Tubes were then centrifuged at 300*g* for 10 min at room temperature to separate the cells and debris. The top part of the supernatant was placed in sterile disposable 2 mL microtubes and all the samples were stored at −20 °C before analysis.

Sample preparation

For endogenous substances, such as melatonin, present at ultra-low levels in biological fluids, the blank biological matrix is obviously not "blank". Also in the commercial TCM 199 medium, melatonin and its oxidative metabolites were present in low levels. For the recovery study, a pool of FF and TCM 199 medium were analyzed in triplicate

LLE was performed as follows: a volume of 450 μL FF was spiked with 15 μL of IS solution (10 000 pg mL^{-1} MEL-d_4 in methanol) and 100 μL of ammonium hydroxide solution (10%, v/v in Milli-Q water). For the TCM 199 medium, a volume of 25 μL was spiked with 15 μL of IS solution (10 000 pg mL^{-1} MEL-d_4 in methanol) and 20 μL of ammonium hydroxide solution (10%, v/v in Milli-Q water) and 200 μL of ultrapure water was added into a labeled 2 mL polypropylene tube and vortexed (2000 rpm) for 2 min. The samples were stored at 8 °C for 10 min. Then 500 μL of extraction solution, methyl *t*-butyl ether/ethyl acetate (1 : 1, v/v), were added and the solution was vortex mixed at 2000 rpm for 3 min following centrifugation at 12 500 rpm for 5 min. The supernatant (±450 μL) was transferred into a labeled polypropylene tube. The extraction procedure was repeated twice in order to obtain a volume of ± 1350 μL, which was evaporated at 45 °C to dryness under a N_2 stream. The FF samples were reconstituted in 150 μL of mobile phase. TCM 199 samples were reconstituted in 300 μL of mobile phase in amber vials. The samples were vortex mixed at 2000 rpm for 2 min following centrifugation at 14 000 rpm for 2 min prior to analysis by HPLC–ESI(+)-MS/MS system.

Results and discussion

This work introduces a method developed and validated for multiple trace compound analyses of melatonin and its metabolites in biological fluids and culture media, which were represented by bovine FF and TCM 199 medium used for oocyte *in vitro* maturation. High analytical confidence was obtained by

Table 1 Optimized MS/MS conditions for determination of melatonin and its oxidative metabolites

Analyte	[M + H]	Type	*m/z*	DP[a]/V	CE[b] /V	EP[c] /V	CXP[d] /V
MEL	233.1	Quantification	130.1	100	59	10	20
		Confirmation	159.1		35	10	8
AMK	237.1	Quantification	114.1	46	17	10	18
		Confirmation	136.1		29	10	28
AFMK	265.1	Quantification	136.0	51	31	10	24
		Confirmation	178.2		17	10	8
6-HMEL	249.1	Quantification	158.0	36	31	10	22
		Confirmation	130.0		51	10	20
MEL-d_4	237.1	PI	133.1	86	17	10	8

[a] DP = declustering potential. [b] CE = collision energy. [c] EP = entrance potential. [d] CXP = collision cell exit potential.

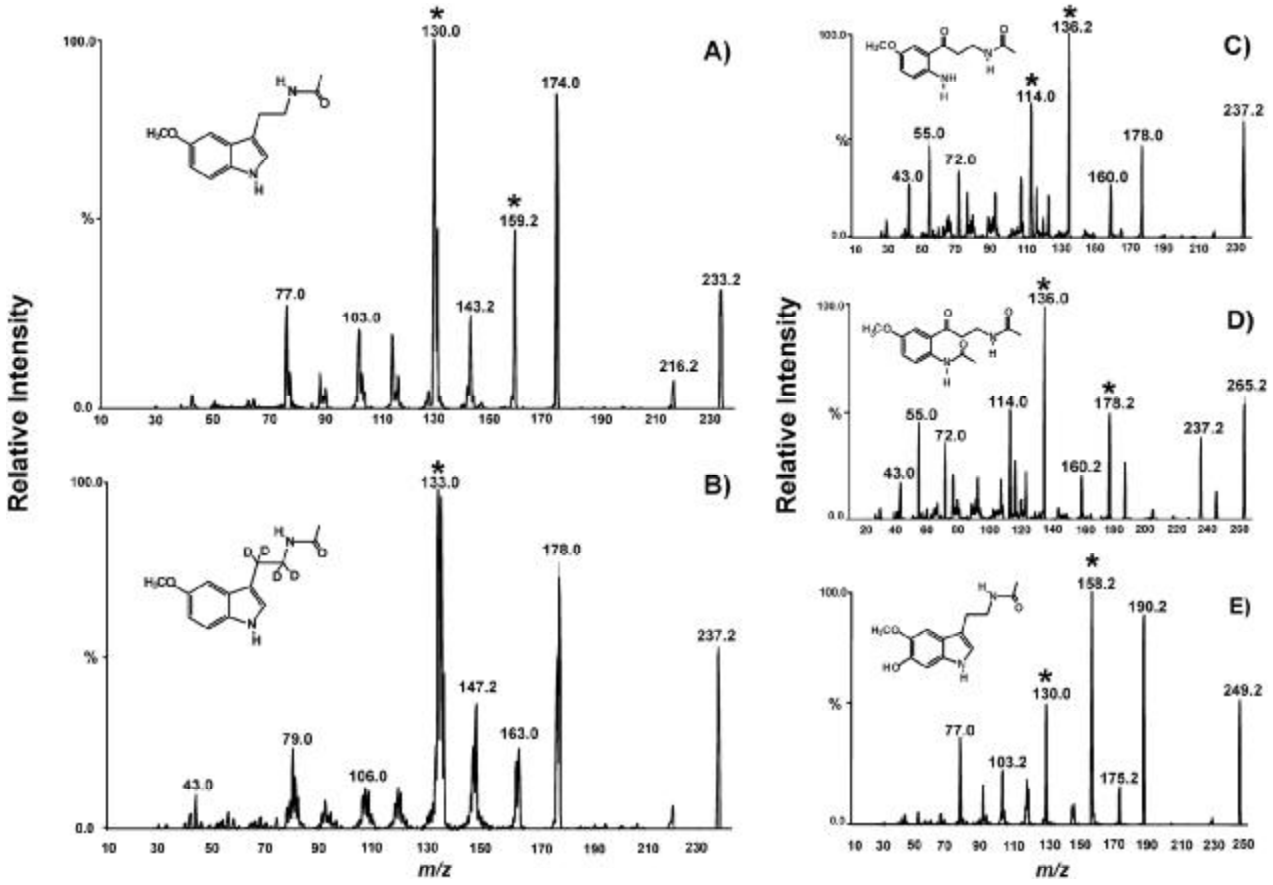

Fig. 1 ESI(+)-MS/MS for CID of the $[M + H]^+$ ions of (A) MEL, (B) MEL-d_4, (C) AMK, (D) AFMK, and (E) 6-HMEL in solvent.

HPLC–ESI(+)-MS/MS using LLE. As already applied in a previous method developed to quantify progesterone in bovine plasma by HPLC–MS/MS,[29] the sample preparation protocol was simple, non-expensive and rapid. Also, quantitation of melatonin and its metabolites could be easily adapted to other biological fluids and media. Data acquisition in the MRM mode was applied for maximum specificity and sensitivity and acquisition parameters were optimized for each compound for best ESI(+) performance.

Melatonin and its metabolites as well as IS (MEL-d_4) were characterized by ESI-MS/MS experiments with direct infusion. All compounds were detected as protonated molecules and MS/MS data, as obtained from CID of the $[M + H]^+$ ions of MEL, MEL-d_4, AFMK, AMK and 6-HMEL, were similar to those previously reported.[30] The main fragment ions correspond to neutral losses of 17 Da (NH_3), 28 Da (CO), 32 (MeOH), 42 Da (CH_2CO), 44 Da (CO_2) and 59 Da (CH_3CONH_2). The MS/MS for $[MEL + H]^+$ of *m/z* 233 displayed fragments ions of *m/z* 216 $[M + H - NH_3]^+$, *m/z* 174 $[M + H - NH_3 + CH_2CO]^+$ or $[M + H - CH_3CONH_2]^+$, and *m/z* 130 $[M + H - NH_3 + CH_2CO + CO_2]^+$ or $[M + H - CH_3CONH_2 + CO_2]^+$. The MS/MS of $[MEL\text{-}d_4 + H]^+$ was similar to that of MEL with the respective 4 Da shift. For $[AFMK + H]^+$ of *m/z* 265, fragment ions correspond to consecutive neutral losses of CO, CH_3CONH_2 and CH_2CO forming ions of *m/z* 237, 178 and 136. $[AMK + H]^+$ fragmentation involved consecutive neutral losses of CH_3CONH_2 and CH_2CO forming ions of *m/z* 178 and 136 respectively; as well as two low abundant fragment ions of *m/z* 124 and *m/z* 114. The MS/MS of $[6\text{-HMEL} + H]^+$ of *m/z* 249 showed consecutive neutral losses of CH_3CONH_2 (*m/z* 190), MeOH (*m/z* 158) and CH_2CH_2 (*m/z* 130). Fig. 1 shows the choices of the MRM transitions for quantitative analysis.

The HPLC method included the use of a column with reduced particle size (2.6 μm) to obtain increased efficiency and high speed as described by Munõz *et al.*[31] Fig. 2 illustrates profiles obtained for each compound. MRM analysis of MEL, 6-HMEL, AFMK and AMK were highly selective with no interfering compounds for both the FF (Fig. 2a) and TCM 199 samples (Fig. 2b). Chromatograms obtained from samples spiked with low levels of the analytes were obtained at 10 min analysis time. The time of the analytical run was defined as 3.5 min (Fig. 2c) for FF and TCM 199.

Linearity, range, carry-over, LOD and LOQ

Bovine FF and TCM 199 medium were not used as matrices for the calibration curve since they contained melatonin and its oxidative metabolites. Consequently, samples used for the calibration curve were prepared in mobile phase solution and the quantitation range was corrected by the recovery of deuterated melatonin. Calibration curves were obtained from

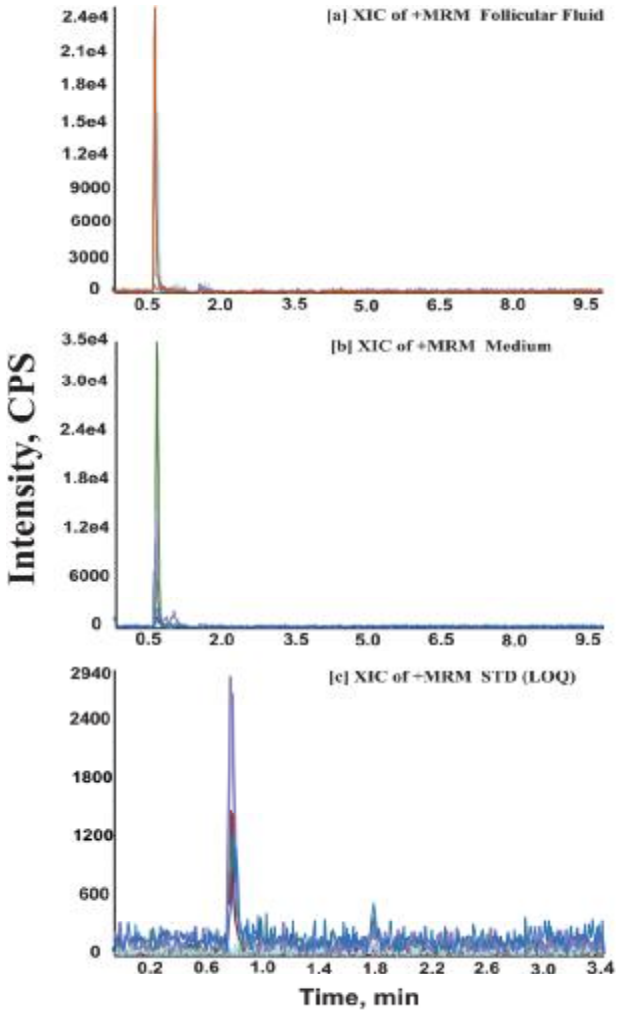

Fig. 2 HPLC–ESI(+)-MS/MS chromatograms in the MRM mode of (a) FF, (b) tissue culture medium (TCM 199), (c) standard spiked in low level of melatonin and its oxidative metabolites in solvent.

the peak area ratio (y) of the analyte to IS against the concentrations of analyte (x) using Multiquant Software Version 1.3.1 (AB Sciex). The correlation coefficient values ($R^2 > 0.999$) indicated appropriate correlations between the investigated compound concentrations and their peak area within the test ranges (Table 2). The analyte 6-HMEL was studied at two different ranges of 100–1500 pg mL^{-1} and 2.5–25 ng mL^{-1} since 6-HMEL may be present at higher concentrations in the samples.

Concentrations of MEL higher than 5 μg mL^{-1} caused column carry-over. The adequate work range to obtain good method performance for MEL, 6-HMEL, AFMK and AMK was 0.005–25 ng mL^{-1}.

The LOD and LOQ signal-to-noise (S/N) values were determined for seven injections of FF and TCM 199 medium samples extracts spiked with MEL (15 pg mL^{-1}), 6-HMEL (150 pg mL^{-1}), AFMK (12.5 pg mL^{-1}) and AMK (12.5 pg mL^{-1}). LOD were calculated based on signals with 3 times signal-to-noise (S/N) values and LOQ were calculated based on 10 times S/N values. LOD and LOQ were found to be, respectively: MEL 3.0/10.0 pg mL^{-1}, AMK 3.0/10.0 pg mL^{-1}, AFMK 3.0/10.0 pg mL^{-1} and 6-HMEL 30.0/100.0 pg mL^{-1} for two types of samples.

Matrix effect

To reduce matrix effects sample extraction with a mixture of TBME and ethyl acetate with 10% ammonium hydroxide solution was performed. The matrix effect was investigated at three different concentrations for melatonin and its metabolites in FF: concentrations of 50, 250 and 500 pg mL^{-1} were used for MEL; 30, 150 and 300 pg mL^{-1} for AFMK; 30, 150 and 500 pg mL^{-1} for AMK and 1500, 3000 and 6000 pg mL^{-1} for 6-HMEL. In TCM 199 for matrix effect estimation, the concentrations were 150, 650 and 1100 pg mL^{-1} for MEL; 90, 800 and 1100 pg mL^{-1} for AFMK/AMK and 50, 160 and 600 pg mL^{-1} for 6-HMEL.

The matrix effect was then investigated by comparing the peak areas of the IS prepared in mobile phase with FF and TCM 199 extracts spiked at the same nominal concentrations. The matrix effects for FF were 2.0, 2.7 and 1.52% for MEL; 3.0, 2.5 and 1.00% for AMK; 2.5, 2.7 and 1.02% for AFMK and 2.0, 2.3 and 1.88% for 6-HMEL. The matrix effects for TCM 199 were 3.2, 2.3 and 1.12% for MEL; 2.8, 1.8 and 0.92% for AMK; 2.4, 1.6 and 0.97% for AFMK and 2.8, 1.5 and 1.02% for 6-MEL. These results show that the ion signal suppression was negligible for the analyte detection as the matrix-matched standards areas were slightly smaller than the mobile phase standards.

Precision, recovery and accuracy

The accuracy of the method was defined by the measured concentration based on a percentage of the expected concentration. Low, medium and high quality control (QC) samples at three different concentrations were injected on the same day to assess the intra-day variation. Inter-day assay variation was assessed by the injection of three samples of each concentration on three days.

The percent relative standard deviation (% RSD) of the regressed (measured) concentrations was used to report precision. The recoveries for all compounds were investigated at three concentration levels and they were between 82% and 108% for FF and from 80% to 106% for TCM 199, respectively. Table 2 reports the intra-day and inter-day variation data (RSD) of melatonin and its metabolites.

Stability (standard solution and autosampler)

No significant degradation (less than 1%) was observed even after a 24 h storage period in the autosampler tray at the final concentrations of melatonin and its oxidative metabolites in FF and TCM 199 when samples were stored at 10 °C. Nonetheless, at room temperature the degradation was higher than 15% after 24 h. The standard solution (150 ng mL^{-1}) diluted in water and stored at −20 °C degraded by 8% in two weeks. The calibration mixtures (100–1500 pg mL^{-1} for 6-MEL and 15–1500 pg mL^{-1} for others compounds) diluted in the mobile phase and then stored at 10 °C for 72 h degraded by 10% for MEL, AFMK and AMK and 15% for 6-MEL. These results established the stabilities defined in the Material and method section.

Table 2 Method performance for melatonin, AMK, AFMK and 6-HMEL in bovine FF and oocyte culture medium (TCM 199)

Analyte	RT[1]	$(R2)^2$	Equation	Linear range	Level FF pg mL^{-1}	Level TCM 119 pg mL^{-1}	$N = 9$ recovery % FF	$N = 9$ recovery % TCM 119	$N = 3$ intraday repeatability RSD (%) FF	$N = 3$ intraday repeatability RSD (%) TCM 119	$N = 9$ interday repeatability RSD (%) FF	$N = 9$ interday repeatability RSD (%) TCM 119
MEL	0.85 ± 0.02	0.99965	$y = 0.00268 \times + 0.04223$	15–1500 pg mL^{-1}	50	150	107	85	0.95	1.89	1.25	3.16
					250	650	90	81	1.28	0.94	2.27	1.45
					500	1100	82	86	1.43	3.10	2.35	3.45
AMK	0.84 ± 0.02	0.99956	$y = 0.00221 \times + (-) 0.01317$	12.5–750 pg mL^{-1}	30	90	107	106	1.27	0.92	1.92	1.12
					150	800	105	80	1.34	1.53	2.47	2.43
					500	1100	97	99	0.36	1.15	1.00	1.60
AFMK	0.84 ± 0.02	0.99962	$y = 0.00156 \times + (-) 0.01325$	12.5–750 pg mL^{-1}	30	90	108	101	0.98	2.27	3.55	2.74
					150	800	82	95	1.09	0.25	2.30	1.14
					300	1100	88	84	0.32	0.96	1.24	2.17
6-HMEL	0.82 ± 0.02	0.99951	$y = 1.02460 \times 10^{-4}x + 1.76099 \times 10^{-4}$	100–1500 pg mL^{-1}	1500	50	88	106	1.86	1.26	2.35	2.72
		0.99962	$y = 1.84112x + 1.85027$	2.5–25 ng mL^{-1}	3000	160	97	94	0.94	0.88	1.43	2.47
					6000	600	90	88	1.87	1.74	2.51	2.41

Analysis of the bovine FF and TCM 199 samples

As already mentioned, several methods to quantify melatonin and its metabolites have been described,[32–35] but to our knowledge, this is the first method directly applied for the quantification of melatonin and its metabolites by LC–MS/MS in a biological fluid and in cell culture medium represented by the bovine FF and TCM 199 (Table 3). In order to apply the method in a relevant biological question, two FF pooled samples of each size, small (<6 mm) and large (>8 mm), from bovine ovarian follicles were aspirated separately from bovine ovaries collected at a commercial abattoir and two samples of TCM 199, 0 h and 24 h after exposure of medium to culture conditions were analyzed. An increase of the AMK concentration with ovarian follicle growth was observed for the four FF pooled samples. For the TCM 199 samples, results showed a decrease in MEL and an increase in AMK concentration after 24 h with variation of the components over time, probably due to exposure to culture conditions. This fact may bring opportunities to test different culture conditions in order to investigate which concentrations and/or exposure periods would be the best for cells to benefit from the cytoprotective effect of melatonin and/or its metabolites. These results are in agreement with the hypothesis of the importance of MEL metabolism during culture.[36–38] As recent studies have shown, melatonin is important for maintaining the integrity of the reproductive system and previously unknown functions have been lately reported.[39] The method herein developed can be further applied to unravel more specific roles of melatonin and its active metabolites, which are much less studied in this and other biological models. Specifically for mammalian reproduction, LC–MS/MS can be applied in the monitoring of melatonin and its metabolites during follicular growth and also to compare dominant and subordinate follicles, so as to determine their behaviors. This fundamental information would allow the development of culture conditions that could better mimic what occurs *in vivo*. Few studies have shown that melatonin varies in the FF according to follicle growth in pigs[40] and humans[41] and its inclusion in cultures may have beneficial impacts.[42–44] The high sensitivity and selectivity of the method would allow the analysis of small amounts of samples, which is particularly common in studies with humans, increasing the probability of gathering information not only on melatonin, but also to uncover the possible contributions of its metabolites for reproductive health.

Table 3 Analysis of samples of small (<6 mm) and large (>8 mm) bovine FF and tissue culture medium (TCM 199) under culture conditions at 0 h and 24 h

Sample			MEL/pg mL^{-1}	6HMEL/ng mL^{-1}	AMK/pg mL^{-1}	AFMK/pg mL^{-1}
FF	<6 mm	(A)	43 ± 0.40	1.32 ± 0.050	59 ± 0.60	70 ± 0.30
	<6 mm	(B)	73 ± 1.5	1.23 ± 0.060	71 ± 0.40	69 ± 0.70
	>8 mm	(C)	53 ± 0.40	1.02 ± 0.030	42 ± 0.90	53 ± 1.1
	>8 mm	(D)	47 ± 1.0	1.06 ± 0.030	51 ± 0.70	44 ± 1.7
TCM 199	0 h	(E1)	708 ± 14	1.29 ± 0.040	191 ± 9.0	340 ± 10
	24 h	(E2)	525 ± 12	1.17 ± 0.040	324 ± 3.0	370 ± 10
	0 h	(F1)	569 ± 2.0	1.22 ± 0.070	440 ± 4.0	430 ± 40
	24 h	(F2)	294 ± 10	1.21 ± 0.010	610 ± 19	450 ± 20

Conclusion

A simultaneous HPLC–ESI(+)-MS/MS methodology able to quantify melatonin and its oxidative metabolites in biological fluid and culture media has been developed and validated using ovarian FF and tissue culture medium as models. The method employs a very simple sample preparation protocol (liquid extraction), requires no derivatization, and displays high sensitivity, selectivity, accuracy and low susceptibility to interferences.

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