Journal of Chromatography B, 877 (2009) 2173–2177

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

A simple and sensitive method for determination of melatonin in plasma, bile and intestinal tissues by high performance liquid chromatography with fluorescence detection

José L.P. Muñoz, Rosa M. Ceinos, José L. Soengas, Jesús M. Míguez*

Departamento de Biología Funcional y Ciencias de la Salud, Facultad de Biología, Universidad de Vigo. 36310 Vigo, Spain

ARTICLE INFO

Article history:
Received 2 February 2009
Received in revised form 21 May 2009
Accepted 1 June 2009
Available online 6 June 2009

Keywords:
HPLC
Fluorescence detection
Melatonin
Plasma
Intestine
Bile

ABSTRACT

This paper describes the development of a simple and sensitive method for routine quantification of melatonin in low sample amounts by using standard equipment of HPLC with fluorescence detection. A double chloroform extraction with an intermediate cleaning step with 0.1N NaOH allowed to concentrate melatonin and to avoid interferences in extracts of the different tissues assayed. The analytical procedure was found to be precise and linear for a wide range of melatonin concentrations. The retention time of melatonin was about 9 min and the recoveries were in the range of 89–94%. The lower limit of quantification estimated on extracted samples was 8 pg/ml. This method was validated in daytime and nighttime samples of plasma, bile and intestinal tissues of trout.

1. Introduction

Melatonin (N-acetyl-5-metoxytryptamine) is produced by the pineal gland and retinal photoreceptors of almost all vertebrates, and it is related to many functions which have in common a rhythmic expression [1,2]. Melatonin is synthesized from 5-hydroxytryptamine by N-acetylation followed by methylation. Is most species, the large increase in the AANAT enzyme at night is the responsible for rhythms of melatonin [3,4].

Melatonin has also been detected in peripheral tissues and fluids of several vertebrate species [5,6]. It has been postulated that the gastrointestinal tract (GIT) is a significant source of melatonin, and might contribute to the blood levels of the hormone [7,8]. Moreover, melatonin has been detected in human bile, liver and portal blood, suggesting that may act as a mediator of inter-organ communication between gut and liver [9]. In addition, GIT melatonin could participate in the regulation of smooth muscle contraction, food intake, transport of electrolytes, and acts as scavenger of free radicals, among others [9,10]. However, few studies have measured melatonin levels in fish GIT and related fluids to assess their relationship with circulating melatonin.

The extremely low levels of melatonin during the daytime, particularly in plasma where the hormone is diluted, have led researchers to develop accurate analytical methods. Most of existent studies have quantified melatonin in pineal, retina and plasma samples by radioimmunoassay (RIA), because of its high sensitivity allows one to outline melatonin's daily changes. Regarding the GIT, melatonin has been quantified by RIA in mammals, but also in fish, amphibians and a reptile [5,6,11]. As an alternative to RIA, GC–LC/MS and HPLC have been successfully used in a number of biological studies to determine melatonin levels ([12], for a review). Although HPLC was previously coupled to electrochemical detection (ED) to improve sensitivity [13–15], oxidation/reduction of melatonin required high electric potentials that originated elevated background currents. A further combination with fluorescence detection (FD) permits the analysis of melatonin without derivatization, being this detection type more versatile to quantify melatonin in complex samples (i.e., plasma, central and peripheral tissues) that need prior extraction and contain low melatonin levels [16–21]. On this basis, the aim of the present study was to develop a simple, sensitive and reproducible FD-HPLC method, capable of responding to the demand for non-expensive analysis of melatonin in samples of body fluids (i.e., plasma, bile) and peripheral tissues (i.e., intestine).

* Corresponding author at: Departamento de Biología Funcional y Ciencias de la Salud, Edificio de Ciencias Experimentales. Facultad de Biología, Universidad de Vigo, 36310 Vigo. Spain. Tel.: +34 986 812386.
E-mail address: jmmiguez@uvigo.es (J.M. Míguez).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.06.001

2. Experimental

2.1. Chemicals

Melatonin was from Sigma (St. Louis, MO, USA). Chloroform and acetonitrile (HPLC grade) were purchased from VWR Prolabo (Fontenay sous Bois, France). All other solvents and reagents were of analytical grade. Stock solutions of melatonin standard were prepared in methanol:water (1:10, v/v) and stored a −4 °C. Working solutions were prepared daily to the desired concentrations.

2.2. Animals and sample collection

Rainbow trout (*Oncorhynchus mykiss*, 99 ± 12 g) were allocated in tanks and acclimated to a 12:12, light:dark cycle (lights on at 08.00 h, 400 lux intensity), with constantly running and aerated water at a temperature of 14 ± 1 °C. Fish were fed with commercial dry pellets. The experiments complied with the Guidelines of the EU Council (86/609/EU), and the Spanish laws (RD 1201/2005) for the use of animals in research.

To obtain blood samples, fish were anaesthetized with MS-222 (50 mg/L^{-1}) and then blood (1 ml) was collected by caudal venous puncture with heparinised syringes. Plasma was obtained after centrifugation of blood (6000 g/10 min) and stored at −20 °C. A series of plasma samples run in parallel were deproteinized by adding 0.2 ml of 0.4 M perchloric acid and centrifuged. Supernatant was buffered to pH 7.4 with sodium bicarbonate and stored at −20 °C.

After sacrifice of fish by decapitation, bile (0.2–0.5 ml) was obtained by puncture of the bile vesicle. The entire intestine was removed and cleaned by the infusion of a volume of NaCl 0.6% (v/v), and segments corresponding to the midgut and hindgut were dissected out. Tissues obtained and bile were stored at −80 °C until assay. To assist sampling during the scotophase a low intensity red light was switched on.

2.3. Extraction of melatonin from plasma, bile and GIT tissues

Measurement of plasma melatonin was preceded by a two-fold extraction procedure with chloroform. Briefly, a 200 μl aliquot of plasma sample was mixed (1:1, v:v) with 0.1 M acetic acetate buffer (pH 4.6) and 2 ml chloroform added. The mixture was vortex-mixed for one min, centrifuged (3800 g, 10 min), and the aqueous phase aspirated. The organic layer was separated and cleaned once with 500 μl 0.1N NaOH. After stirring and posterior centrifugation, the aqueous phase was aspirated and the organic layer was evaporated to dryness under an air stream at room temperature. The residue was dissolved in 100 μl of mobile phase and filtered through 0.5 μm filters. An aliquot (50 μl) of the filtrate was injected into the HPLC system. A similar procedure was followed to extract melatonin from bile except that a sample volume of 100 μl was mixed with an equal volume of acetic acetate buffer and then extracted with 1 ml of chloroform.

For extraction of melatonin from midgut and hindgut, small portions of tissue (30–40 mg) were sonicated in 500 μl of acetic acetate buffer, centrifuged (10,000 × *g*/10 min), and the supernatants were taken for new vials and mixed with 2.5 ml of chloroform (1:5, v:v ratio). The mixtures were centrifuged, the aqueous layers aspirated, and the organic layers were separated and cleaned once by adding 500 μl 0.1 N NaOH. After stirring and centrifugation, the organic layer was dried and residue was dissolved in 100 μl mobile phase, and 50 μl was injected into the HPLC system.

The efficiency of the extraction procedure was determined on samples (plasma, bile, intestine homogenate), stripped by adding 50 mg of charcoal to each ml of sample, stirring for 1 h at 20 °C, and allowing the mixture to sit for 24 h at 4 °C. The solution was then centrifuged at 4000 × *g* for 45 min at 4 °C, and the supernatant filtered twice through a 0.45 μm filter. The stripped samples were spiked with authentic standard solutions of melatonin assayed in triplicate. Linearity of response (calibration curves) between melatonin added to the blank samples and melatonin recovery was evaluated in a range of concentrations of 5–500 pg/ml plasma, and 5–200 pg/ml bile or intestine homogenate. Intra-assay variation was determined in 5 extracted plasma aliquots containing melatonin 50 pg/ml, and analyzed on the same day. Inter-assay variation was determined on eight extracted plasmas spiked with two melatonin concentrations (5 and 50 pg/ml) that were analyzed on different days.

2.4. HPLC apparatus and conditions

Chromatographic system consisted of a Gilson 321 solvent delivery pump equipped with a 50 μl Rheodyne injection valve, and a Jasco FP-1520 fluorescence detector set at 285/360 excitation/emission wavelengths. Melatonin was separated on a Beckmann Ultrasphere ODS column (3 μm particles, 75 mm × 4.6 mm i.d.). The mobile phase consisted of a solution of 85 mM acetic acetate, 0.1 mM EDTA-Na_2, and acetonitrile 14% of final volume, pH adjusted to 4.7. All analyses were performed at room temperature at a flow rate of 1.0 ml/min. Acquisition and integration of chromatograms was performed using the BiocromXP software (Micron Anal., Madrid, Spain). Quantification of sample peaks was done by comparing peak areas with those of standards.

2.5. Statistics

Data are expressed as mean ± standard error of mean (S.E.M.). Melatonin levels in tissues obtained at different time points were analyzed by one-way ANOVA. Statistical was performed with Sigma Stat v3.0 software.

3. Results and discussion

3.1. Sample extraction and melatonin recovery

For the determination of melatonin in blood several extraction procedures have been described to concentrate and eliminate interference from the sample material. The most common ones involved the use of solvents such us dichloromethane and chloroform, and solid-phase extraction (SPE) using cartridges with different packing materials [12]. The SPE extraction has been shown to be a selective procedure, although more expensive than organic solvents, and when used on complex biological matrix (i.e., tissues), it requires prior cleaning steps of the sample in order to avoid the clogging of the column. Also it has been warned also that recovery rate of melatonin from pre-packed SPE column varies among the batches and manufacturer [12]. Alternatively, a liquid–liquid extraction is a non-expensive method and easily adapted to different types of biological samples, i.e. fluids or tissues. In our case, chloroform was chosen instead of dichloromethane since it left lesser residues that could affect chromatographic melatonin peak. A similar chloroform procedure allows us to extract melatonin not only from trout plasma and bile, but also from homogenates of GIT, a tissue that despite its big size posses a low amount of melatonin per unit of tissue mass. In order to improve the accuracy of extraction, we used a double chloroform step with an intermediate cleaning of the organic phase with 0.1N NaOH. This process was very effective in removing non-specific interferences affecting melatonin detection signal. The high efficiency of the extraction procedure allowed recovery values for spiked melatonin to samples assayed of 86–98% (Table 1).

Table 1
Recovery values for the extraction of melatonin from plasma, bile and intestinal tissues of rainbow trout.

[Mel] added (pg/ml fluid or tissue homogenate)	Plasma (total)	Deproteinized plasma	Intestinal tissue	Bile
5	94.2 ± 8.0	92.1 ± 6.0	86.1 ± 6.1	87.5 ± 3.7
10	96.3 ± 4.6		90.2 ± 7.1	
50	92.6 ± 3.9	91.6 ± 4.1	91.8 ± 4.5	93.0 ± 2.7
100	98.5 ± 6.9		88.6 ± 3.7	
200	98.8 ± 2.3	93.6 ± 5.4	89.5 ± 5.3	94.6 ± 5.6
500	95.3 ± 4.8			

Recovery values express the percentage of melatonin measured after adding a known amount of melatonin to samples previous to extraction and quantification. Data are mean ± SEM of three samples for each concentration. Linear regression equations for plasma and intestine spiked with melatonin were as follows: $y = 0.9399x + 8.1365$, $r^2 = 0.992$ (plasma) and $y + 0.8872x + 1.2964$, $r^2 = 0.989$ (intestine).

3.2. Chromatographic assay optimization and quality

Even though ED has been postulated to display a higher sensitivity than native FD to measure indole-derivative compounds [12], in our experience ED has de disadvantage of the high potentials needed to oxidize/reduce melatonin [4]. The high currents originated tend to disturb the stability of the chromatogram baseline which often results in lower reproducibility. On the other hand, we observed that the use of organic solvents for the extraction of complex samples (like plasma or bile) contributed to deteriorate peak resolution and sensitivity, often resulting in slow assays. This problem was avoided by cleaning chloroform with NaOH before fluorescence detection.

The elution profiles obtained for the melatonin standard and the different types of samples assayed are depicted in Figs. 1 (plasma) and 2 (intestine, bile). No interfering peaks with the separation and identification of melatonin were observed at the retention time in any of the samples assayed. The use of columns with a reduced particle size (3 μm) and length (7.5 cm) provided increased efficiency, a short analysis time and reduced solvent costs. In this sense, an advantage of the technique was the relatively low organic content in the mobile phase needed to elute melatonin (14% acetonitrile), as compared with other methods for melatonin measurement that use the same organic solvent [13,17] or methanol [14–16,19,21]. The retention of melatonin from the C_{18} stationary phase was 9–10 min, and could be further shortened by using mobile phases with increasing percentages of organic solvent.

Both retention time and peak area values gave a good reproducibility determined by the low intra-assay (3.19%) and inter-assay (4.13%) coefficient of variation. Likewise, an excellent linear relationship ($r \sim 0.99$) was found between the concentration of melatonin added to charcoal-stripped samples and the amount of melatonin recovered after extraction (Table 1). Meanwhile, the limit of detection (LOD, based on a signal to noise ratio of 3:1) was 12 pg/ml for unextracted aqueous standards, and the lower limit of quantification (LLOQ), was 8 pg/ml for extracted (and concentrated) fluid/tissue samples. The sensitivity of this method was similar to that showed in other highly sensitive HPLC procedures [13,15–17,21] but, as an advantage, it reduces further the sample amount (i.e., 200 μl plasma) needed for quantification, which is frequently a limit when collecting samples from small-size experimental animals.

3.3. Quantitative analysis of samples

Quantification of plasma melatonin levels is the most feasible parameter targeted to evaluate changes in hormone production in the body. Inspite or this, most of reported HPLC methods were developed to measure melatonin content in the pineal organ, which is considered as the main source of body melatonin [1,4]. However, plasma levels of the hormone are very low, in particular at daytime, and only a few methods have been reported to measure it. In addition, several of them involved complex processes of sample enrichment or derivatization in order to improve sensitivity [18–20], which difficult its suitability for a large number of laboratories. In this way, our method allows direct and reliable measures of the lowest circulating melatonin levels (Table 2), which were consistent with those showed in trout by chromatographic methods [16] and also by RIA [22]. The near to three-fold increase at night with respect to daytime values found in plasma melatonin also agreed with previous studied in trout [22,23,26].

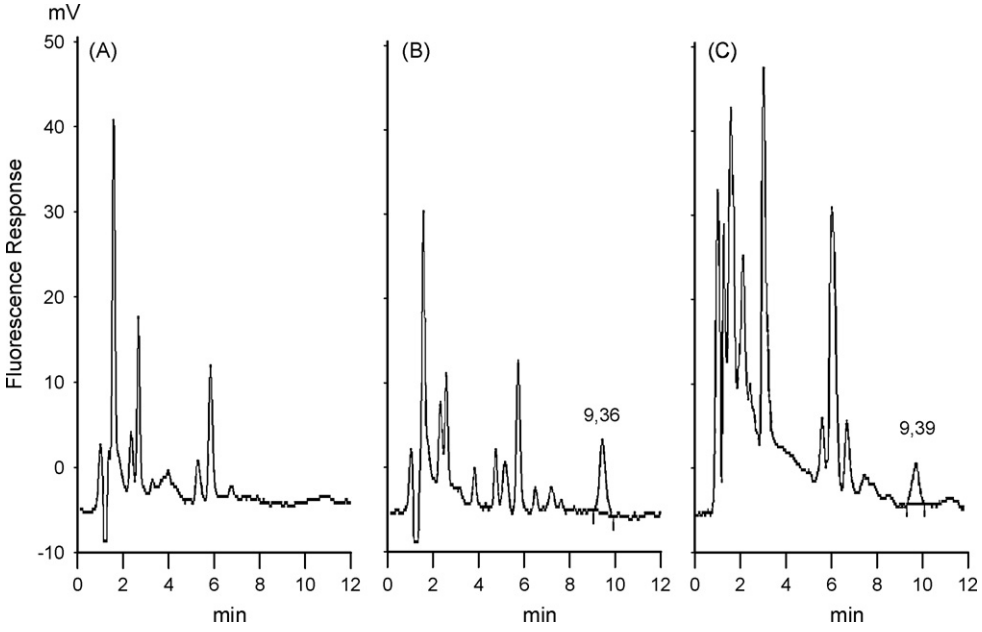

Fig. 1. Determination of melatonin in trout plasma after chloroform extraction. Chromatograms represent (A) a charcoal-stripped plasma sample, (B) the same sample spiked with melatonin at a concentration of 166.5 pg/ml plasma, (C) an extract of trout plasma obtained during daytime (value of melatonin obtained was 88.7 pg/ml plasma).

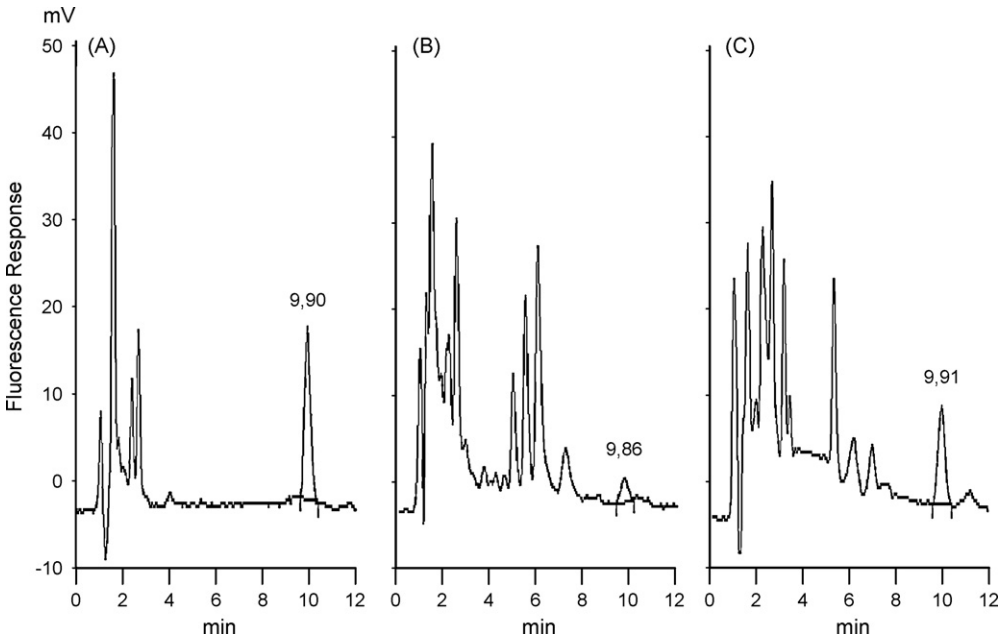

Fig. 2. Chromatograms of extracts of (A) a melatonin aqueous standard (50 pg on column), (B) a sample of intestine (midgut), (D) a sample of bile. Values of melatonin were 117.3 pg/g in midgut, and 1783 pg/ml in bile.

A high percentage of blood melatonin (about 70%) is bound to proteins, and reliable measures of the circulating hormone should include detachment of bound melatonin to be part of the free plasma fraction. Otherwise, measures made on untreated plasma samples could underestimate melatonin values [17]. With the present method we have found similar melatonin contents in free-protein plasma and total plasma samples, indicating that the extraction with chloroform was able itself to remove bounded melatonin. Therefore, the method has the advantage that a deproteinization of plasma samples is not really required, which also benefits from undesirable sample dilution and the presence of precipitates that could interfere with assays.

The GIT has been also proposed as an important source of melatonin in vertebrates [5,7]. Moreover, in mammals melatonin content is higher in portal than in peripheral blood [6], suggesting that melatonin diffuses easily through membranes and blood vessels reaching entero-hepatic circulation. Our data also show that melatonin could be locally synthesized in most of intestinal length of trout, which agrees with previous evidence in this species [24,25]. The overall amount of melatonin formed in trout GIT (160–260 pg/g tissue) could be relevant to contribute to plasma values of the hormone, particularly during the daytime. It is also noteworthy the absence of significant day–night changes in the content of melatonin both in midgut and hindgut, which agrees with previous studies in several teleost species [26].

Melatonin levels in bile did not show significant day–night differences and varied among 1259–1550 pg/ml, which is near to 10-fold the values of plasma during daytime and 3-fold those found at night (Table 2). These high values agree well with the idea that melatonin might concentrate in bile [27], showing one of the highest concentration of the body [7,27]. Although the function of such a high concentration is not known, a protective role of the entero-hepatic melatonin in GIT mucosa has been proposed [10]. Measurements of the hormone levels at these locations will aid to understand its role in digestive-metabolic physiology. Here we described a single and fast analytical method which enables the quantification of melatonin in small fluid volumes and intestinal tissues. This method made easier and unexpensive procedures for a routine analysis of melatonin

Table 2
Day–night profiles of melatonin concentrations in total and deproteinized plasma, bile and homogenates of intestinal tissues (midgut and hindgut).

	Day (12–14 h)	Night (23–01 h)
Protein-free plasma (pg/ml)	103.1 ± 2.0 (n = 15)	453.9 ± 31.7* (n = 15)
Total Plasma (pg/ml)	123.1 ± 18.8 (n = 15)	422.1 ± 36.2* (n = 15)
Bile (pg/ml)	1259.8 ± 123.4 (n = 8)	1550.5 ± 91.4 (n = 8)
Midgut (pg/g tissue)	262.1 ± 31.6 (n = 10)	218.6 ± 38.4 (n = 10)
Hindgut (pg/g tissue)	161.6 ± 32.6 (n = 10)	174.3 ± 32.3 (n = 10)

Values are expressed as mean ± S.E.M. The units (pg/ml, pg/g) and number of samples assayed are indicated in parenthesis.
* $P < 0.05$ night *vs.* day plasma values, ANOVA.

Acknowledgements

This work was supported by grants AGL2007-65744-C03-01 from MEC (Spain). José L. P. Muñoz was recipient of a Doctoral fellowship from CONICYT (Chilean Government).

References

[1] R.J. Reiter, Experientia 49 (1993) 654.
[2] J. Falcón, Trends Endocrinol. Metabol. 18 (2007) 81.
[3] D.C. Klein, P.H. Roseboom, S.L. Coon, Trends Endocrinol. Metab. 7 (1996) 106.
[4] R.M. Ceinos, S. Rábade, J.L. Soengas, J.M. Míguez, Gen. Comp. Endocrinol. 144 (2005) 67.
[5] G. Huether, Experientia 49 (1993) 665.
[6] G.A. Bubenik, S.F. Pang, J.R. Cockshut, P.S. Smith, L.W. Grovum, R.M. Friendship, R.R. Hacker, J. Pineal Res. 28 (2000) 9.
[7] G.A. Bubenik, R.R. Hacker, G.M. Brown, L. Bartos, J. Pineal Res. 26 (1999) 56.
[8] M. Messner, G. Huether, T. Lorf, G. Ramadori, H. Schwörer, Life Sci. 69 (2001) 543.
[9] G.A. Bubenik, Digest. Diseases Sci. 47 (2002) 2336.
[10] S.J. Konturek, P.C. Konturek, T. Brzozowski, G.A. Bubenik, J. Physiol. Pharmacol. 58 (2007) 23.
[11] G.A. Bubenik, S.F. Pang, Gen. Comp. Endocrinol. 106 (1997) 415.
[12] T. Harumi, S. Matsushima, J. Chromatogr. B 747 (2000) 95.
[13] R. Vieira, J.M. Míguez, M. Lema, M. Aldegunde, Anal. Biochem. 205 (1992) 300.
[14] T. Harumi, H.G. Akutsu, S. Matsushima, J. Chromatogr. B 675 (1996) 152.
[15] E. Chanut, J. Nguyen-Legros, C. Versaux-Botteri, J.H. Trouvin, J.M. Launay, J. Chromatogr. B 709 (1998) 11.
[16] E. Kulczykowska, P.M. Iuvone, J. Chromatrogr. Sci. 36 (1998) 175.
[17] V. Rizzo, C. Porta, M. Moroni, E. Scoglio, R. Moratu, J. Chromatogr. B 774 (2002) 17.

[18] L. Hamase, J. Hirano, Y. Kosai, T. Tomita, K. Zaitsu, J. Chromatogr. B 811 (2004) 237.
[19] E. Bechgaard, K. Lindhardt, L. Martinsen, J. Chromatogr. B 712 (1998) 177.
[20] F. Iinuma, K. Hamase, S. Matsubayashi, M. Takahashi, M. Watanabe, K. Zaitsu, J. Chromatogr. A 835 (1999) 67.
[21] J. Sastre-Toraño, P. van Rijn-Bikker, H.J. Merkus, Guchelaar, Biomed. Chromatogr. 14 (2000) 306.
[22] W.A. Gern, D.W. Owens, C.L. Ralph, Gen. Comp. Endocrinol. 34 (1978) 453.
[23] T. Masuda, M. Iigo, K. Mizusawa, M. Naruse, T. Oishi, K. Aida, M. Tabata, Zool. Sci. 20 (2003) 1011.
[24] B. Fernández-Durán, C. Ruibal, S. Polakof, R.M. Ceinos, J.L. Soengas, J.M. Míguez, Gen. Comp. Endocrinol. 152 (2007) 289.
[25] O. Lepage, E.T. Larson, I. Mayer, S. Winberg, J. Pineal Res. 38 (2005) 264.
[26] E. Kulczykowska, H. Kalamarz, J.M. Warne, R.J. Warne, J. Comp. Physiol. B 176 (2006) 277.
[27] D.X. Tan, L.C. Manchester, R.J. Reiter, W. Qi, M. Hanes, N.J. Harley, Life Sci. 62 (1999) 2523.