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Determination of melatonin in rat pineal, plasma and retina by high-performance liquid chromatography with electrochemical detection

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Abstract

A sensitive method for the routine measurement of endogenous melatonin (MEL) in pineal, retina and plasma rat tissues has been developed using reversed-phase high-performance liquid chromatography with electrochemical detection. Quantification limit for MEL was 0.2 ng/mg protein in pineal, 15 pg/ml in plasma and 2.0 pg/mg protein in retina. To improve both MEL quantification and the reproducibility of the assay, an internal standard was used when an extraction in organic solvent was required, in contrast with other available chromatographic methods. MEL values and the circadian profile obtained in this study from both rat pineal and plasma agree with those reported previously. This method allows MEL detection in mammal retina, particularly in rat, where MEL levels are very low. © 1998 Elsevier Science B.V.

Keywords: Rat retina; Melatonin

1. Introduction

Melatonin (*N*-acetyl-methoxytryptamine, MEL) is a neurohormone produced and released in vertebrates by pinealocytes of the pineal and photoreceptor cells of the retina under the control of light and darkness exposure (circadian rhythm). MEL mediates physiological, endocrinological and behavioural processes [1]. The synthetic pathway is identical in both tissues, successively involving serotonin-*N*-acetyltransferase (NAT), which converts serotonin (5-HT) into *N*-acetyl-serotonin (N-Ac-5-HT), and hydroxyindole-O-methyltransferase (HIOMT) leading to MEL. The rate-limiting enzyme, NAT, is stimulated during dark exposure; MEL is thought to be a chemical transducer of the light/dark message [2]. MEL secreted by the pineal is rapidly released in the blood stream; MEL concentrations in blood are higher at night compared to diurnal level. MEL from pineal plays several roles in photoperiodic species such as reproduction and circadian rhythm coordination [3]. In humans, it is implicated in various disorders such as seasonal depression and anxiety

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[4]. Exogenous MEL has been shown to resynchronise free-running circadian rhythm in experimental animals and the sleep/wake cycle in humans, and to improve the sleep onset and quality in jet-lag and shift-work. MEL seems also to be implicated in immune system responsiveness [5]. Finally, hydroxyl radical scavenger properties of MEL have been described, suggesting its role in the aging process [6].

In contrast to pineal MEL, retinal MEL acts as a local paracrine effector of dark adaptative responses, such as photoreceptor outer segment disc renewal and their phagocytosis by the retinal pigment epithelium [7,8].

Analytical methods developed to quantitate endogenous MEL in various tissues and fluids can be divided into three groups. The immunological assays and particularly radioimmunoassay (RIA) are widely used for pineal, plasma or serum MEL quantification because highly sensitive and easy to use commercial kits are available [9–13]. However, there is evidence that the antibodies against melatonin used in these kits can crossreact with still unidentified but related components in some biological samples, calling into doubt the specificity of such tests. Moreover, each sample used in RIA can only be used for MEL determination; other compounds such as MEL precursors or metabolites have to be quantified in other samples using other analytical procedures. Another approach, using gas chromatography–mass spectrometry (GC–MS), after derivatisation of the compounds, has been developed [14,15]. It can achieve equal levels of sensitivity to RIA, however, its use on a routine basis is not easily accessible because of costly apparatus and maintenance. The last group of methods uses the liquid chromatography (LC) approach, with either electrochemical [16–20] or fluorescence [21–24] detection. MEL in pineal is easy to quantify due to its high concentration compared to the detection limit achieved. In addition, the sample preparation is relatively simple. Thus, the proposed methods are relatively easy to use. In contrast, quantitation of MEL in plasma is far more complex, due to the low level of the hormone in the samples, and requires an extensive preparation of the sample with an extraction step to concentrate MEL. However, the currently proposed methods do not involve the use of an internal standard. The internal standard, whose analytical behaviour is quite similar to that of MEL, added in controlled amount in the sample at the first step of the procedure, is highly recommended to limit interassay variability. In addition, no chromatographic methods have been described for assay of MEL in retina, particularly in mammal retina where retinal concentrations are very low, compared to lower vertebrates such as birds.

The aim of this work was to develop an LC method, using electrochemical detection (LC–ED), which allows the routine assay of MEL in various biological samples such as pineal, plasma and retina, whose preparation has been adapted according to the level of quantification to be achieved. Using this method, physiological data on MEL content in rat pineal and plasma, and interaction between dopamine (DA) and MEL in rat retina have been studied.

2. Experimental

2.1. Apparatus and chromatographic conditions

The chromatographic system consisted of an isocratic pump (P100, Thermo Separation Products, Les Ulis, France), an autosampler (ISS 100, Perkin Elmer, St-Quentin-en-Yvelines, France) equipped with a 7125 Rheodyne injection valve and a 100-μl injection loop. The column was a reversed-phase C_{18} (150 mm×4.6 mm, 5-μm particle size, Ultrasphere Beckman, Gagny, France). The mobile phase consisted of water–acetonitrile (Fisons) (80:20, v/v) containing 0.01 m*M* EDTA, 0.1 m*M* KH_2PO_4 and 0.5 m*M* octane sulfonic acid (Pic B8, Waters, St-Quentin-en-Yvelines, France). The pH was adjusted to 4.70, and the mobile phase filtered through HVLP filters (0.45 μm, Millipore). The flow-rate was 1.3 ml/min. Electrochemical detection was performed with an EG and G model 400 amperometric detector (EG and G, Evry, France) at a working potential set at +900 mV (glassy carbon electrode), relative to an Ag/AgCl electrode. Integration of the chromatographic peaks was performed using a Shimadzu CR 5A module (height percent method) (Touzart and Matignon, Les Ulis, France).

2.2. Methods

2.2.1. Melatonin extraction from biological samples

2.2.1.1. *Pineal*. Preparation of the pineal was adapted depending on the analytical assay to be used. For LC–ED assay, individual pineal was sonicated in 50 μl of 0.2 *M* perchloric acid containing 0.1% $Na_2S_2O_5$ (Merck) and 0.1% EDTA (Merck) using a Vibra-cell microsonicator (20 s, 20 kHz, 40 W). Homogenates were then centrifuged (5 min, 4000 *g*, +4°C). The clear supernatants were stored at −80°C before injection (20 μl) into the LC system. Resulting pellets were used for protein determination (see below). For RIA assay, pineals were treated as previously described [25–27] and diluted 2000-, 3000- and 5000-fold before assay.

2.2.1.2. *Plasma*. Plasma samples (1 ml) were added with 5 ml of dichloromethane (CH_2Cl_2) after alkalinisation (100 μl 1 *M* KOH) and addition of 10 μl of 6-fluoro-tryptamine (0.05 μ*M*, Sigma) as internal standard. The extraction was performed by horizontal shaking for 10 min. After centrifugation (10 min, 1500 *g*, +4°C), the aqueous layer was removed by aspiration and the organic layer evaporated under nitrogen. The dry residue was dissolved in 100 μl of mobile phase and immediately injected (50 μl) into the LC system. Extraction recovery was determined by spiking known quantities of MEL (50, 100, 250, 500, 1000 pg) to daytime (MEL level is below the limit of detection) plasma aliquots (1 ml) from adult male Wistar rats. Spiked samples were treated as previously described.

2.2.1.3. *Retina*. Pools of eight retina were homogenised in 500 μl of ice-cold 0.2 *M* phosphate buffer (PBS, pH 6.5). The homogenate was then extracted (shaken for 10 min) with 5 ml of CH_2Cl_2 after alkalinisation (100 μl 1 *M* KOH) and addition of 10 μl of 6-fluoro-tryptamine (0.05 μ*M*) as internal standard. After centrifugation (10 min, 1500 *g*, +4°C), the supernatant aqueous phase and the remaining pellet at the interface were removed for protein quantification, and the organic layer was evaporated under nitrogen. The dry residue was dissolved in 100 μl of mobile phase and directly injected (60 μl) into the LC system. Extraction recovery was determined by spiking known quantities (50, 100, 250, 500, 1000 pg) of MEL to pools of eight retina collected during daytime (endogenous MEL undetectable) from adult male Wistar rats. The spiked samples were then treated as previously described.

2.2.2. MEL quantification

2.2.2.1. *Pineal*. For LC–ED assay, the electrochemical detector response was found to be linear in the range 20 to 2000 pg of MEL injected onto the column. To take into account the response variability of the detector during the assay, a standard solution of MEL (1000 pg/20 μl) was injected every other five consecutive samples. MEL content was calculated using the MEL peak height in the analysed sample compared to the mean peak height of the two bordering standard solutions. For RIA, a standard curve was established using (in duplicate) six different concentrations of unlabelled MEL (from 242 to 8.5 fmol in 2-fold dilution steps).

2.2.2.2. *Plasma, retina*. A linear relationship was established between MEL content and peak height ratio (MEL/internal standard) in the range 25 to 1000 pg MEL injected. In these conditions, MEL content was calculated using the peak height ratio in each sample compared to a standard curve (50 to 1000 pg of MEL). The standard curve analysis (6 points, in duplicate) was carried out every day.

2.2.3. DA and DOPAC assays

Retinal DA and DOPAC concentrations were determined using an LC–ED method, previously described [28].

2.2.4. Protein quantification

The amount of protein in pineal and retina was determined in the pellets (see above) using the Bradford method [29], and bovine serum albumin as standard.

2.2.5. Statistical analysis

The comparison between the RIA and LC–ED

results was analysed using the Student *t*-test.

For statistical comparison in the animal experiments, data were analysed using a global Kruskal–Wallis analysis, followed, when significant ($p<0.05$), by individual intertreatment comparisons according to the nonparametric Mann–Whitney U-test.

2.3. Animals

2.3.1. General conditions

Animals were kept at 22–23°C under standard conditions with a 12-h light–dark cycle (lights on/off at 7 a.m./7 p.m.) and free access to food and water for 2 weeks before use. Any steps of the experiment (rat handling, blood collection, tissue dissection) scheduled during the dark phase of the light–dark cycle were carried out under dim red light. The sampling time and tissue collection were as follows:

2.3.2. Analytical comparison: MEL assay in pineal

Twenty Wistar male rats (220±25 g, Iffa Credo, France) were decapitated between 10:30 and 11:00 p.m.. Pineals were removed, quickly frozen on dry ice and stored at −80°C until assay. They were randomly distributed for RIA ($n=10$) or LC–ED assay ($n=10$).

2.3.3. Time course of MEL in pineal and plasma

Thirty-six Wistar male rats (220±25 g, Iffa Credo, France) were used. To study the MEL concentrations in plasma at various time points during the night period, the 12-h light–dark cycle was inverted (lights on/off at 7 p.m./7 a.m.) for 3 weeks before the experiment. Decapitation occurred during the dark phase (local time 08:00 a.m.–06:00 p.m.) and blood was collected in heparinised tubes. Pineals were removed, quickly frozen on dry ice and stored at −80°C until assay. Blood was centrifuged immediately (15 min, 1200 *g*, 4°C). Plasma was separated and stored at −80°C until assay.

2.3.4. Pharmacological study: MEL assay in retina

Brown–Norway male rats (200±25 g, Janvier, France) were randomly allocated ($n=12$ per group) to the following treatments, injections being performed by i.p. route (2 ml/kg): α-methyl-*para*-tyrosine (AMPT, Sigma) was dissolved in saline, and animals received a first injection (150 mg/kg) and a second injection (250 mg/kg), at 30 and 1 h before killing, respectively. Clozapine (Sandoz) was dissolved in a minimal amount of HCl and then diluted in water (final pH 5.5–6). Animals received 30 mg/kg, 1 h before killing. Raclopride (tartrate, Astra), dissolved in water, was administered at 1.5 mg/kg, 1 h before killing. Control animals received a single injection of saline, 1 h before killing. In each group, rats were decapitated between 11:00 and 11:30 p.m. (local time, no inversion of the light cycle). Eyes were rapidly enucleated, hemisected and vitreous removed. Retina were dissected out and quickly frozen on dry ice. In each group, they were pooled by eight (i.e. four animals per pool, three pools of retina per treatment) and stored at −80°C until assay.

3. Results and discussion

3.1. Chromatographic assay validation

For MEL quantification in pineal, the homogenisation step in perchloric acid was enough to allow a 99–100% recovery of MEL in the perchloric phase. In addition, due to the very simple procedure (direct homogenisation and centrifugation, no organic extraction), no losses were foreseen. Therefore, the use of an internal standard was deemed not necessary. This was however further confirmed in a parallel comparison; the presence of an internal standard did not improve the precision of MEL determination by LC–ED in pineal (data not shown). Fig. 1 shows a chromatogram of an external standard (A) and of a pineal sample (B), after perchloric extraction.

In contrast, as far as MEL quantification in plasma or retina was concerned, because of the potential losses of MEL during organic extraction, it was decided to use an internal standard to improve the reproducibility of the assay. Using the extraction protocol described above, the recovery of MEL was 96–98% and 94–97% from plasma and retina, respectively. To our knowledge, this is the first time an internal standard was used for MEL quantification in chromatographic assays. In addition, the interest for using an internal standard was supported by the

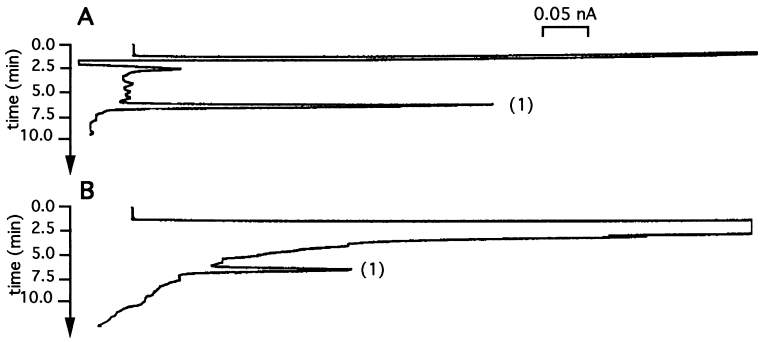

Fig. 1. (A) Chromatogram of a standard solution containing 500 pg of MEL, directly injected on to the column. (B) Chromatogram of a Wistar rat pineal gland collected at night (11.00 p.m). (1)=MEL peak. The mobile phase consisted of 0.1 *M* potassium phosphate, 0.5 m*M* octane sulfonic acid, 0.01 m*M* EDTA and 20% acetonitrile (v/v). pH 4.7. Flow-rate 1.5 ml/min. Applied potential 900 mV.

decrease from 19 to 11% of the within coefficient of variation for MEL quantification, when MEL is extracted from standard solutions (50 to 500 pg) in the presence of the internal standard (Table 1). Fig. 2 shows chromatograms of a standard solution (A), a plasma sample (B) and a retina sample (C), after dichloromethane extraction.

In terms of precision, for evaluation of the within- and between-assay coefficients of variation (C.V.) for MEL quantification, samples of pooled pineal and pooled plasma at low and high concentrations were prepared and analysed three times on the same day (within C.V.) and on four different days (between C.V.). The C.V. values were never greater than 15% (Table 1). Such coefficients were not evaluated for retina as a pool of retina could only be used for one determination, thus the total number of retina whould be too large for those calculations.

The limit of detection (LOD) of MEL was calculated to be 8 pg MEL (34 fmol) injected on the column, using a 3:1 signal-to-noise ratio (see typical background noise on Fig. 1 or Fig. 2A). The limit of quantification (LOQ) was calculated on the same approach of signal-to-noise ratio comparing signals from samples with known low concentrated samples to those of blank samples. For a signal-to-noise ratio of 5:1, LOQ was estimated to be 0.2 ng/mg protein in pineal (no organic extraction step), whereas in plasma and retina (with an organic extraction step), LOQ was estimated at 15 pg/ml and 2.0 pg/mg protein, respectively, bearing in mind that for retina, it is necessary to pool at least eight retina to achieve such a detection.

In order to assess the specificity of the LC–ED system, it was checked whether other compounds in the analysed samples were retained by the column or electroactive at +0.90 V potential and particularly that tryptophan, *N*-acetyl-serotonin, 5-HIAA, and 5-HT (the major compounds present in pineal) as well as catechole compounds such as DA and its metabolites (present in retina) were not coeluted and codetected with MEL in our chromatographic and

Table 1
Within- and between-assay coefficients of variation (C.V.)

MEL concentrations	Extracted standard (pg/ml)		Pineal (ng/mg protein)		Plasma (pg/ml)	
	50	500	0.5	20	50	250
Within C.V. (%)[a] (n=3)	11	8	9	8	12	8
Between C.V. (%)[a] (n=4)	15	12	11	9	15	12

[a] Value from *n* determinations.

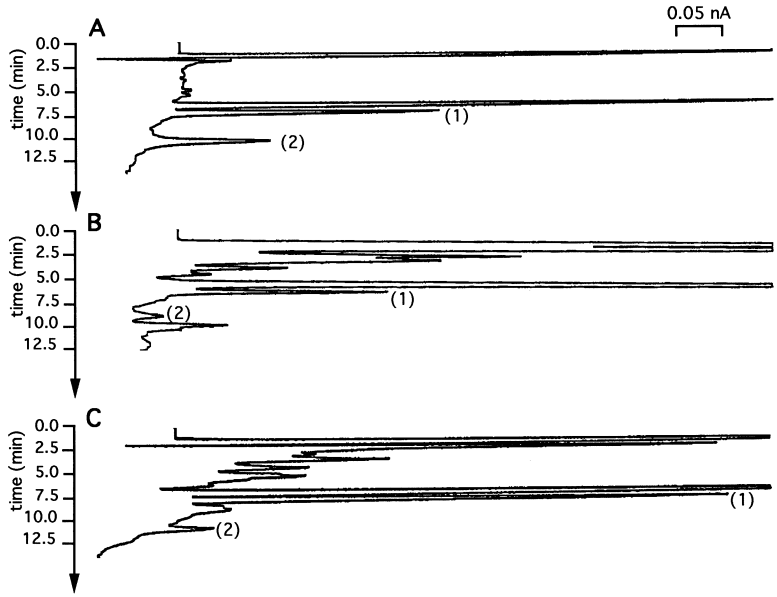

Fig. 2. (A) Chromatogram of a standard solution of MEL and 6-fluoro-tryptamine as internal standard, obtained after alkalinisation and extraction with dichloromethane (typical example from point 250 pg of the standard curve; for experimental conditions, see Section 2). (B) Chromatogram of a Wistar rat plasma (corresponding to a MEL concentration of 75 pg/ml plasma) collected during the dark phase (inverted light cycle, local time 08:00 a.m.–06:00 p.m.). (C) Chromatogram of a pool of eight Brown–Norway rat retina (corresponding to a MEL concentration of 21 pg/mg protein) collected between 11:00 and 11:30 p.m. (local time, no inversion of the light cycle). (1) internal standard peak, (2) MEL peak. Other peaks are from the background noise of the extract (blank run not shown). Analytical conditions were the same as in Fig. 1, except that the flow-rate was set at 1.3 ml.

detection conditions. All these compounds did not interfere with MEL detection in the described system. It is noteworthy that these compounds can be separated and analysed using the same apparatus but different chromatographic conditions, as described previously [28].

3.2. LC–ED versus RIA results

MEL concentrations measured in 20 nocturnal pineal samples using either the LC–ED or the RIA assay were not significantly different (mean±S.D.: 1.33±0.18 and 1.27±0;18 μg/mg protein for LC–ED and RIA, respectively). Consequently, LC–ED appears to be a performing and sensitive enough alternative to RIA, making use of a currently available laboratory methodology. In addition, whereas MEL determination from a pineal sample can be achieved in this LC–ED system, the same perchloric extract can also be used for detection and quantification of other compounds of interest, such as indoles, using another chromatographic system. The same holds true for plasma; an aliquot can be used for MEL determination, another for catechole determination, both being analysed using an LC–ED assay. For retina MEL determination, because of the very low level of MEL, it is necessary to use the complete extracted sample, nothing being left for any other analysis. However, the same analytical methodology can be used for other determinations such as indole, catechole or amino acidergic compounds.

In contrast, RIA needs its own unique sample preparation, hence the same sample cannot be used for other determinations. This leads to difficulties when correlations between several parameters in a given tissue are sought. Finally, the LC–ED approach is versatile enough to be used for many other

purposes. It is far less costly than RIA and does not require the use of radioelements.

3.3. Animal studies

Fig. 3A shows the time course of MEL levels in the pineal during a nycthemeral period. An increase in MEL level during the dark period, reaching a maximum in the middle of the night was observed. Both the profile and the endogenous levels observed are in agreement with the MEL circadian rhythm previously described in rats, whatever the assay methodology used [17,19,23,24].

Fig. 3B shows the profile of MEL plasma concentrations during the nychtemer. These values agree with those reported previously [11]. Since MEL is rapidly released from the pineal gland once it is produced, the blood MEL rhythm reflects the amount being produced in the pineal at virtually the same time. Thus MEL concentrations in plasma are also higher at night than they are during light-period. It should be noted that the reported relatively high standard deviation is related to an important animal intervariability and not to the intrinsic analytical variability, as confirmed by repeated assays of plasma from the same animal. As expected, the level of MEL in retina was undetectable during day light, whatever the rat strain used. In contrast, during the nocturnal period, MEL became detectable and even quantifiable in pigmented rat retina. However, this detection was achievable only when retina were pooled. For albino rats, MEL remained undetectable even in a sample of 12 pooled retina.

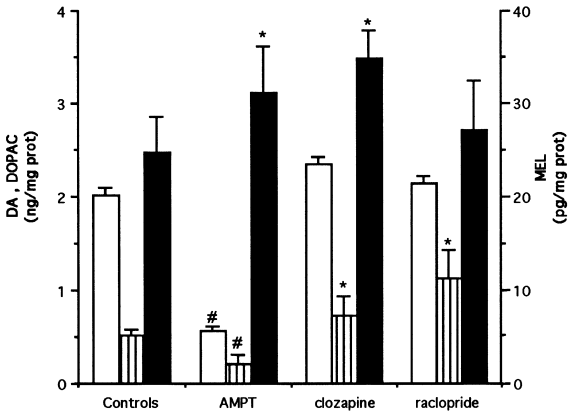

Fig. 4. Effects of α-methyl-*para*-tyrosine, clozapine and raclopride on DA (white), DOPAC (lined) and MEL (black) Brown–Norway rat retinal concentrations * $p<0.05$, # $p<0.01$ versus controls. Control values (mean±S.E.M.) were as follows: DA, 2.01±0.01 ng/mg protein; DOPAC, 0.51±0.07 ng/mg protein; and MEL, 24.7±3.0 pg/mg protein.

Fig. 4 reports the effects of dopaminergic drugs

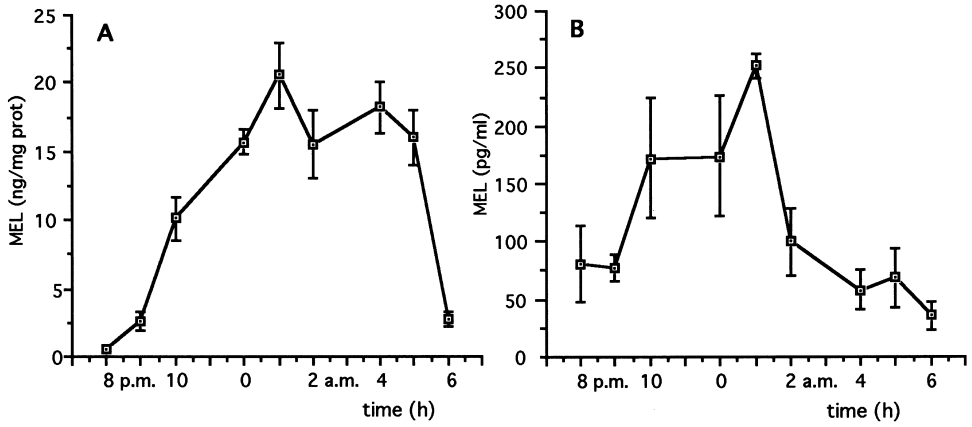

Fig. 3. Time course of MEL concentrations in Wistar rat pineal (A) and plasma (B) (mean±S.E.M., $n=9$ determinations) during the dark period (inverted light cycle, local time 08:00 a.m.–06:00 p.m.).

on DA, DOPAC and MEL in retina. DA and DOPAC were followed in this study to validate the pharmacological tools. Indeed, a 72% ($p<0.01$) decrease in DA levels after DA synthesis inhibition, and an increase in DOPAC levels after receptor blockade (42 and 120% with clozapine and raclopride, respectively, $p<0.05$) were observed. MEL concentrations were increased after both AMPT, an inhibitor of DA synthesis (26%, $p<0.05$) and clozapine, an antagonist of D2/D4 dopaminergic receptors (41%, $p<0.05$), whereas raclopride, an antagonist with D2/D3 affinity had no significant effect. These results suggest that the inhibition of retinal NAT by DA occurs in nonmammalian species as was previously described [30]. The results suggest also that the dopaminergic receptors involved in MEL synthesis are mainly of the D4 subtype. Other investigations related to the localization of D2/D4 dopaminergic receptors on photoreceptor cells have also been performed and confirm this observation [31].

In conclusion, our results indicate that MEL can be adequately quantitated in rat biological samples, including retina, using a highly sensitive, specific and reproducible LC–ED assay. This method can allow to carry on with the investigation of melatoninergic function, particularly in mammal tissues where MEL levels are lower than those in lower vertebrates. For example, in vivo NAT or HIOMT activities, or interactions between MEL and other monoaminergic or amino acid systems will be evaluated, after administration of various pharmacological drugs, or after modification of circadian rhythms.

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