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High melatonin solubility in aqueous medium

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Abstract: The pineal hormone melatonin (5-methoxy-N-acetyltryptamine) has been reported to participate in important physiological processes. Although some of its biological actions seem to depend on a protein receptor at the membrane surface, melatonin is known to interact with a large variety of tissues and cells, suggesting that the molecule may not necessarily interact through a specific membrane receptor at a specific cell. Most discussions of melatonin activity have assumed that the molecule is highly hydrophobic. Contrary to belief, the present work shows that melatonin is soluble in a purely aqueous medium up to 5×10^{-3} M and describes a new method of melatonin preparation which shows the high hydrophilicity of the molecule. The results presented will affect the current biological hypothesis on the need of a melatonin carrier in the blood stream or the mechanisms which allow the hormone to cross the cell membrane and interact at the level of the nucleus.

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Introduction

The complex role of a molecule in the body is related to many factors, including its lipophilic or hydrophilic character. Many aqueous soluble hormones do not penetrate cells but interact at the cell membrane surface with protein receptors. On the other hand, steroid and thyroid hormones, for instance, are known to enter target cells to exert their effects [Stryer, 1988].

Melatonin (5-methoxy-N-acetyltryptamine), a tryptophan-derived hormone, has been reported to interact with a variety of different cells [Reiter, 1991], playing a number of distinct physiological functions, including many photoperiod-dependent responses [Reiter, 1980; Bartness and Goldman, 1989; Armstrong, 1989]. Recently melatonin was shown to behave as a very potent oxygen radical scavenger [Tan et al., 1993a; Reiter et al., 1993], and its anti-aging, life-prolonging [Pierpaoli et al., 1991; Reiter, 1992], and DNA protective effects [Tan et al, 1993b] were suggested to be related to its antioxidant capacity. Although some melatonin membrane receptors have been identified [Morgan and Williams, 1989; Sudgen, 1992], it has been suggested that there could be many melatonin actions where a protein receptor would not be necessary [Reiter et al., 1993].

Melatonin is a small non-charged molecule, which is considered to be highly hydrophobic, possibly due to its chemical structure, but mainly due to the difficulty of directly dissolving melatonin in aqueous media. In the literature, melatonin has always been reported to be prepared by first dissolving melatonin powder in an organic solvent, such as ethanol or dimethylsulfoxide, followed by dilution with an aqueous medium. Because of its presumed hydrophobic nature it was suggested that melatonin requires plasma proteins to be transported in the blood [Cardinali et al., 1972], and it easily crosses the lipid membrane barrier [Reiter, 1992]. Considering its accepted high degree of hydrophobicity, there still remained the question of how melatonin gets into the aqueous compartment of the cell and interacts at the nuclear level [Tan et al., 1993b]. The present work shows that melatonin is soluble in pure aqueous medium up to 5×10^{-3} M when it is prepared by the new method described here.

Materials and methods

Chemicals

Melatonin (P6414, lot 98F8374), purchased from Sigma Chemical Co., St Louis, MO, USA, was employed without further purification. HEPES was from INLAB, Brazil.

Melatonin preparation

Melatonin was prepared based on the method used for the preparation of lipid vesicles [Lasic, 1992].

TABLE 1. Solubility and stability of melatonin in water

Theoretical[1] (M)	Immediately after preparation[2] (M)	3 days after[2] (M)	10 days after[2] (M)	12 days after[2] (M)
2×10^{-5}	1.9×10^{-5}	1.9×10^{-5}	1.9×10^{-5}	1.9×10^{-5}
4×10^{-5}	3.8×10^{-5}	—	—	—
5×10^{-5}	4.5×10^{-5}	—	—	—
1×10^{-4}	8.7×10^{-5}	—	8.3×10^{-5}	—
2×10^{-4}	1.9×10^{-4}	1.9×10^{-4}	1.9×10^{-4}	—
4×10^{-4}	3.9×10^{-4}	3.7×10^{-4}	3.7×10^{-4}	—
5×10^{-3}	4.8×10^{-3}	4.7×10^{-3}	4.7×10^{-3}	4.7×10^{-3}
1×10^{-2}	8.2×10^{-3}	8.2×10^{-3}	5.9×10^{-3}	4.4×10^{-3}
2×10^{-2}	1.7×10^{-2}	7.6×10^{-3}	4.5×10^{-3}	5.1×10^{-3}

[1]Melatonin concentrations calculated from the stock solution (see text).
[2]Melatonin concentrations calculated from optical measurements (see text). Solutions were kept at 4°C.

About 0.3 ml of a 10^{-2} M stock solution of melatonin in chloroform was slowly evaporated under a stream of N_2, while gently spinning the test tube until a dried and homogeneous film was obtained on the bottom of the tube. Residual solvent was removed by evaporation under vacuum for at least 3 hr. Melatonin was then dissolved in water, with vortexing, to the desired final concentration. Results obtained using 10×10^{-3} M HEPES buffer, pH 7.4 were identical to those obtained with water.

Whenever the "traditional method" of melatonin preparation is mentioned here it refers to the technique of dissolution melatonin powder in ethanol (10^{-2} M) and dilution to the desired concentrations with water or buffer, with not more than 0.2% residual ethanol, in volume.

Optical measurements

Melatonin optical absorption spectra were registered in a Aminco SW-100 spectrophotometer, at room temperature, with a 1cm path length quartz cell. For concentrations above 4×10^{-4} M, the samples were diluted before absorption measurement. All the absorption values shown here are mean values of at least 3 samples. Fluorescence emission spectra ($\lambda_{excitation}$ = 278 nm) were registered in a Hitachi 3010 spectrofluorimeter, at room temperature, with a 1cm path length quartz cell.

Biological activity measurements

The toad *(Bufo ictericus)* skin bioassay was utilized as previously reported [Ferroni and Castrucci, 1987]. Isolated skin in physiological saline is light in color due to the melanin granule aggregation within melanocytes. In skin previously darkened by the pigment dispersing agonist α-MSH (α-melanocyte-stimulating hormone), melatonin causes a reversal back to the original light color [Filadelfi and Castrucci, 1993]. The changes in color were monitored by a Photovolt reflectometer and calculated as percentage of the initial (basal) values.

Results and discussion

The optical (240 nm < λ < 300 nm) and fluorescence (300 nm < λ < 400 nm) spectra of melatonin prepared by the new method described here are identical to those obtained with samples made by the traditional method. Signals were registered with melatonin concentrations up to 4×10^{-4} M and 2×10^{-5} M, for optical and fluorescence spectroscopies, respectively.

For higher melatonin concentrations, Table 1 compares the concentration that should be obtained if all the melatonin molecules in the film had been solubilized by water, calculated from the stock solution (first column), with the actual concentration of melatonin in solution, calculated by measuring the samples optical absorption assuming $\epsilon = 6300$ $M^{-1} cm^{-1}$ at λ = 278nm (second column) (Merck Index, 1989). The stability of several melatonin solutions, for up to 12 days, is also shown in Table 1. It can be seen that within an experimental error of 8%, melatonin is soluble in water (or HEPES buffer) up to the concentration of 5×10^{-3} M; furthermore, melatonin is highly stable when kept at 4°C. This result is also emphasized in Figure 1, where it is evident that, for concentrations higher than 5×10^{-3} M, melatonin will precipitate down to its solubility concentration.

Melatonin solubilized by the new method was also checked for biological activity (see Materials and methods). The results obtained were identical to those with the hormone prepared by the traditional method, within the experimental error (Fig. 2).

It was clearly shown here that melatonin is rather soluble in aqueous medium when it is prepared from

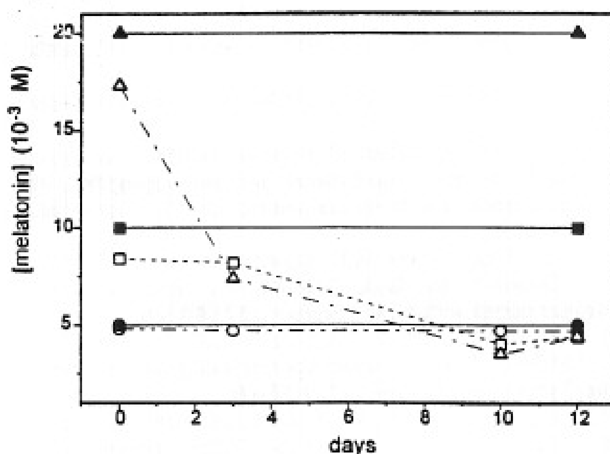

Fig. 1. Time stability of melatonin in water solutions. 2×10^{-2} M, ▲ and Δ; 1×10^{-2} M, ■ and □; 5×10^{-3} M, ● and ○. Full symbols correspond to theoretical values, as explained in the text.

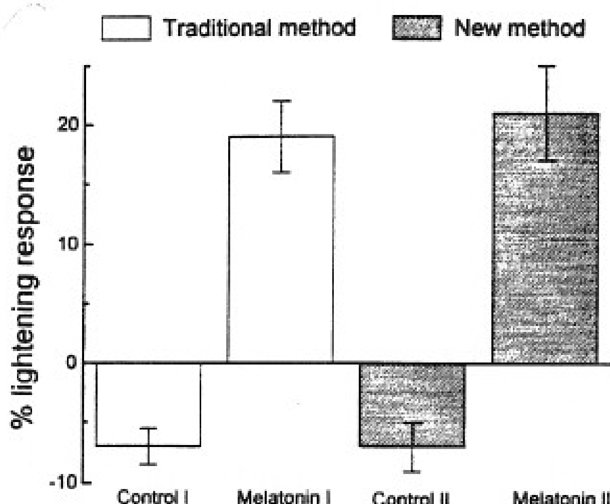

Fig. 2. Lightening effects of melatonin solubilized by the new method as compared to the hormone solubilized by the traditional method in the in vitro toad skin bioassay (n = 6).

a thin film on a glass surface. This is understandable considering that melatonin is an indoleamine where the pyrrole ring can be polarized so the N-H goup may participate in a hydrogen bond with a water molecule. Moreover, the heterocyclic indole could also form hydrogen bonds with water, similar to the benzene ring, as has been recently reported [Suzuki et al., 1992]. Therefore, the role of the film seems to be to make the melatonin molecule more accessible to water, compared to the packed structure of the powder microcrystals.

Another important aspect of the melatonin preparation described here is the possibility of eliminating ethanol from melatonin solutions. Although fluorescence and optical absorption spectra, as well as the biological activity assay described here, showed identical results for the two different melatonin preparations, it has been found that even low concentrations of ethanol (0.1% in volume) exhibit remarkable inhibitory effects on proliferation of cultured neoplastic melanocytes (data not shown).

Though the results above were obtained with melatonin in pure water or HEPES buffer, it is obvious that the melatonin solubility in the different aqueous media of the body will be around 10^{-3} M, which is much higher than the concentration required for the molecule to act. On the other hand melatonin has also been found to partition in lipid vesicles [C.S. Shida et al., in preparation], indicating that this molecule could penetrate a lipid bilayer and bath every part of the cell, possibly locating preferentially in the nucleus [Menendez-Pelaez et al., 1993], and acting, for instance, as an endogenous radical scavenger [Reiter et al., 1993; Poeggeler et al., 1993].

The method of melatonin preparation shown here should be tested with other endogenous molecules or pharmaceutical products that have been considered "water insoluble." The understanding of the solubility properties of biologically relevant molecules is certainly fundamental for the discussion of their role in organisms.

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