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ORIGINAL ARTICLE

A novel observation: Melatonin's interaction with malondiadehyde

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Abstract

OBJECTIVES: A purpose of this study is to determine whether melatonin, a novel antioxidant, can interact with malondialdehyde in an *in vitro* condition.
METHODS: The reaction kinetics and thermodynamics of this reaction have been performed and a new product has been identified using liquid chromatography onlining with mass spectrometry.
RESULTS: A new product generated with an interaction of melatonin and malondialdehyde has been identified to be a three-ring structure with a molecular weight 286, which has the specific wavelength absorption with the maximum at 345 nm. The reaction was completed within a few minutes and the product was proportional to the concentrations of the reactants. Based on its molecular weight and the deduced structure of the new product, we speculate that this reaction occurs between one melatonin molecule and one malondialdehyde and to form a 3 ring conjugated adduct.
CONCLUSION: This is the first report to show that melatonin directly interacts with malondialdehyde to form a new product. The findings suggest that melatonin may detoxify unsaturated carbonyls and protect against cellular damage induced by oxidative stress.

Abbreviations:
MDA, malondialdehyde
TMP, 1,1,3,3-tetramethoxypropane
AGE, advanced glycation end-product

Introduction

One of the important indexes of most stresses, diseases and the aging process is the consistent increase of various toxic carbonyls in almost all tissues and body fluids [1–3]. Following daily activities, particularly after intense exercise or sleep deprivation, the level of toxic carbonyls becomes largely increased [4, 5]. Malondialdehyde (MDA), as one of the most important intermediates of free radical damages [4], is a highly reactive unsaturated carbonyl that can readily bind or crosslink important biomacromolecules such as structural and functional proteins and nucleic acids [4]. It has a half-life of a few hours [6] and thereby can diffuse from the place of generation to other sites to bring further oxidative stress. Such carbonyl stress-related reactions, simple to say carbonylation, can

form unstable and reversible 1:1 amino-carbonyl (Shiff-base) compounds at an early stage of protein modification [4,7]. Though the Shiff-base formation is slow, and can be actively reversed by several defense systems, the accumulation of amino-carbonyl reaction continues following daily activity (e.g. binding and blocking the active sites on neurotransmitter receptors). When the neurons in brain and central nerver system cannot tolerate further interference of carbonylation, a need of rest or sleep may thus required [8].

Melatonin, the major hormone produced by the pineal gland, exhibits characteristic diurnal rhythm of synthesis and secretion at a higher level at night and a lower level during daytime [9]. It has been considered as an endocrine of time-regulator [9] and has been used as a practical therapy for jet lag and disturbances of sleep [10]. Although it is regarded as an antiaging or antioxidative molecule [11], however, what physiological function could melatonin most possibly perform in relation to sleep biochemistry is still uncertain. Since Tan *et al* reported that melatonin can directly scavenge free radical in 1993 [12], it has been widely proved that melatonin is an effective free radical scavenger and can stimulate several antioxidative enzymes [13–15]. While melatonin's antioxidative effect was found ubiquitous, its de-carbonylation after acute free radical damages, probably during sleep, has not been carefully investigated.

In this paper, we report for the first time that melatonin and serotonin in certain conditions reacted readily with MDA to form stable products that may add understanding about the biological functions of melatonin in human health.

Materials and methods

1. Materials and stock solutions

Melatonin (N-Acetyl-5-methoxytryptamine), purity > 99.0%, was obtained from Sigma Chem. Co. (St. Louis, MO, USA). Serotonin creatinine sulfate monohydrate and 1,1,3,3-tetramethoxypropane (TMP) were purchased from Fluka Chemie AG (Buchs, Switzerland). Other chemicals used were all of analar grade.

The melatonin stock solution (10mM) was prepared with an ethanol/water system. Thus, 0.116g melatonin was first dissolved in 1.0 ml absolute ethanol, and then made up to 50.0ml with ultra pure water (from a Milli-Q Academic A10 system). The stock solution was stored in refrigerator at 0–4°C for no longer than two weeks.

Serotonin stock solution (10mM) was directly prepared with ultra pure water, namely, 0.207g serotonin complex was dissolved to 50ml ultra pure water.

A fresh MDA stock solution (50mM) was prepared by hydrolyzing 1,1,3,3-tetramethoxypropane (TMP), which was modified according to a method described by Kikugawa et al. [16]. The concentration of stock solution was decided to avoid possible self-polymerization of MDA in the reaction condition. Thus 0.84ml (5mmol) TMP was mixed with 1.0ml 5.0M HCl and 3.16ml ultra pure water, and shaken at 40 for obtaining MDA through acidic hydrolysis. After the solution became homogeneous, indicating a successful hydrolization of TMP, the stock solution then was made up to 100ml with ultra pure water.

2. Equipment

The Perkin-Elmer Lambda Bio-40 UV/VIS Spectrometer (USA) was used for estimating the absorption spectra of reactions.

The mass spectrometer was an LCMS-2010 single quadrupole equipped with electrospray ionization interface (Shimaduz). The data were collected and processed by using a LCMSSolution software.

3. Experiment conditions

3.1 Spectrophotometry conditions. Absorption maxima of melatonin (233nm, ε=27,550; and 278nm, ε=6,300), and serotonin (275nm, ε=15,000) were confirmed as reported in literature. Absorption maxima of MDA (245nm, ε=13,700 in acid; 267nm, ε=31,500 in neutral or alkaline conditions) were also frequently checked before experiments. 2.0mM of each reactant was chosen as the optimum reaction concentration. Appropriate measurement concentration was at 0.10mM, thus 0.15ml reaction solution was diluted with 2.85ml ultra pure water in cuvette.

3.2 Chromatographic conditions. Chromatographic separations were performed by using a Thermo Hypersil-Keystone Hypurity C18 (150mm × 2.1 mm, 5 μm) analytical column. The oven temperature was set at 35°C. The mobile phase consisted of acetonitrile–10mM NH_4Ac (40:60, v/v) containing 0.2% acetic acid and was isocratically eluted at a flow rate of 0.2 ml/min. The wavelength of the SPD-M10Avp diode array detector was set in the range of 200–350 nm.

3.3 Mass spectrometer conditions. An LCMS-2010 quadrupole mass spectrometer was interfaced with an electrospray ionization (ESI) probe (LC/MS-ESI). Since melatonin can obtain readily a proton in the acidic electrospray ionization conditions [17], the positive electrospray ionization mode has been the choice of detection. The temperatures were maintained at 250°C, 250°C, and 200°C for the probe, CDL, and block respectively. The voltages were set at 4.5 kV, −30V, 25V, 150V, and 1.5kV for the probe, CDL, Q-array 1, 2, 3 bias, Q-array radio frequency (RF) and detector respectively. The flow rate of nebulizer gas was 4.5 L/min. The ions of selection monitoring were decided by positive scanning from *m/z* 50–600. For the quantification of glibenclamide, the analysis was carried out in selection ion monitoring in positive ion mode at *m/z* 233 (melatonin) and 287 (new product). Tuning of mass spectrometer was performed with the help of autotuning function of LCMSSolution software (Version 2.02) using tuning standard solution (polypropylene glycol). Optimization and calibration of mass spectrometer were achieved with autotuning.

Results

Incubation of instantly prepared MDA at 37°C with either melatonin or serotonin resulted in a new absorption peak at about 345nm which indicated clearly that a new product was produced in the incubation

system (Fig. 1). Original absorbance of MDA (245nm), melatonin (223 and 278nm) and serotonin (218 and 275nm) all decreased after reaction. Particularly, the absorbance of MDA disappeared almost completely following reaction.

Time courses of MDA reacting with melatonin and serotonin were shown in Fig. 2. Absorbance at 345nm was measured after 0.5, 2, 4, 8, 12 and 24h of incubation. The reaction product between MDA and melatonin reached the highest value after about 8h and reduced gradually (Fig. 2A). Reactions between MDA and serotonin continued until 24h (Fig. 2B). A time-dependent discoloration, however, was observed in the MDA+melatonin reaction system. Comparing Fig. 2A with 2B, the reaction of MDA with melatonin was notably faster than with serotonin.

Fig. 3 shows a concentration relevance of the new products and each reagent. MDA concentration was seen directly proportional to the new product. The product absorption also increased with the concentration of melatonin (Fig. 3A) as well as serotonin (Fig. 3B).

Reaction temperature has also an important effect on the formation of reaction product and reaction speed. Fig. 4 shows that the best reaction temperature for MDA with melatonin was at about 37°C whereas the best reaction temperature for MDA with serotonin was higher.

According to the absorption spectra given above, the reaction product and the reaction process were proposed based on the knowledge of organic and physical

Figure 1. New products formed from the reaction of MDA with melatonin or serotonin. MDA (2mM) was incubated with melatonin (2mM) or serotonin (2mM) in aqueous solution for 0.5h, at pH 1.5, 37°C. Data are a representative of three reproducible tests.

Figure 2 (A and B). Time course of MDA reacting with (A) melatonin or (B) serotonin. A sample of 2mM MDA was incubated with 2mM melatonin or 2mM serotonin. The incubations were carried out at 37°C in aqueous solution, pH 1.5. Data were a representative of several tests.

Figure 3 (A and B). MDA reacted with melatonin or serotonin at different concentrations. Reaction at 37°C, pH 1.5. Absorbance (345nm) of reaction solutions was measured after 4h. Different concentrations of MDA were reacted with 0.25, 0.5, 1, 2, 4 and 8mM melatonin (A) or serotonin (B). Data were a representative of several tests.

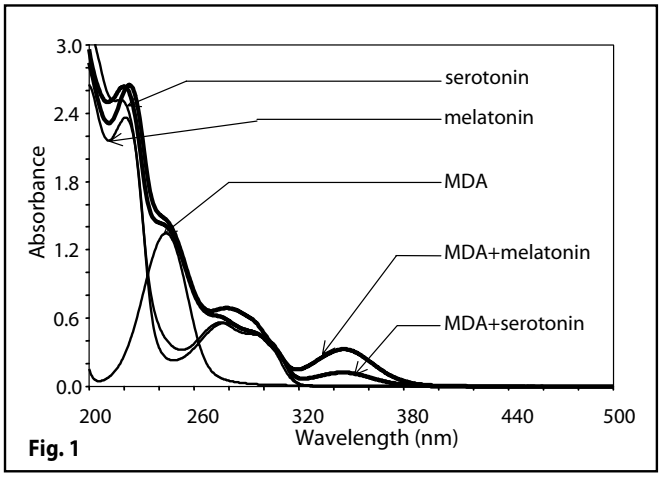

Fig. 1

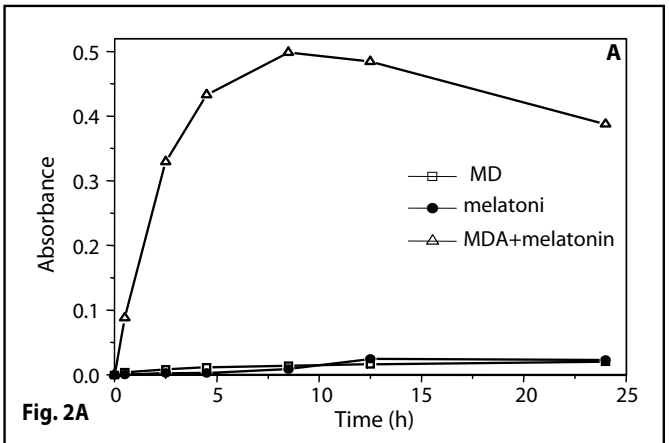

Fig. 2A

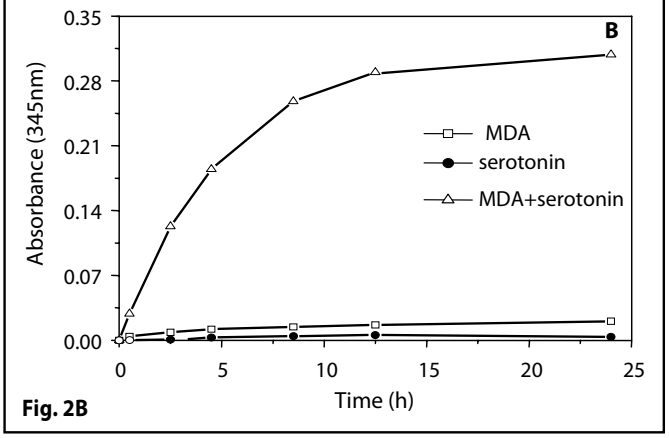

Fig. 2B

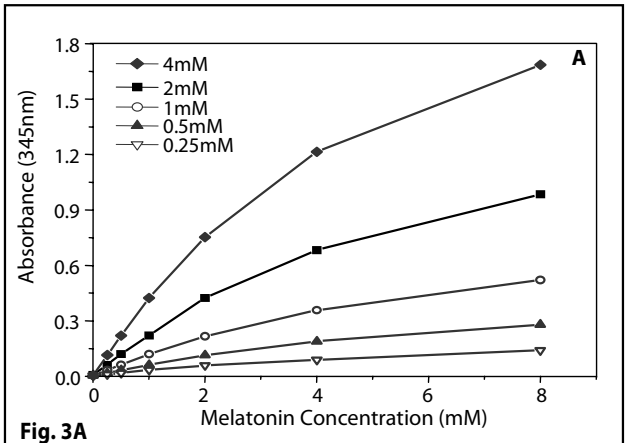

Fig. 3A

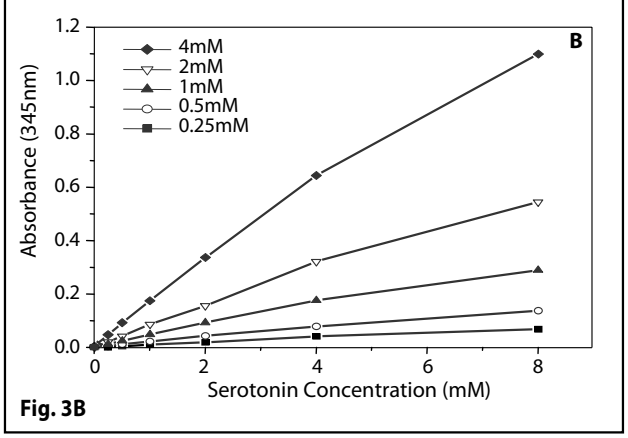

Fig. 3B

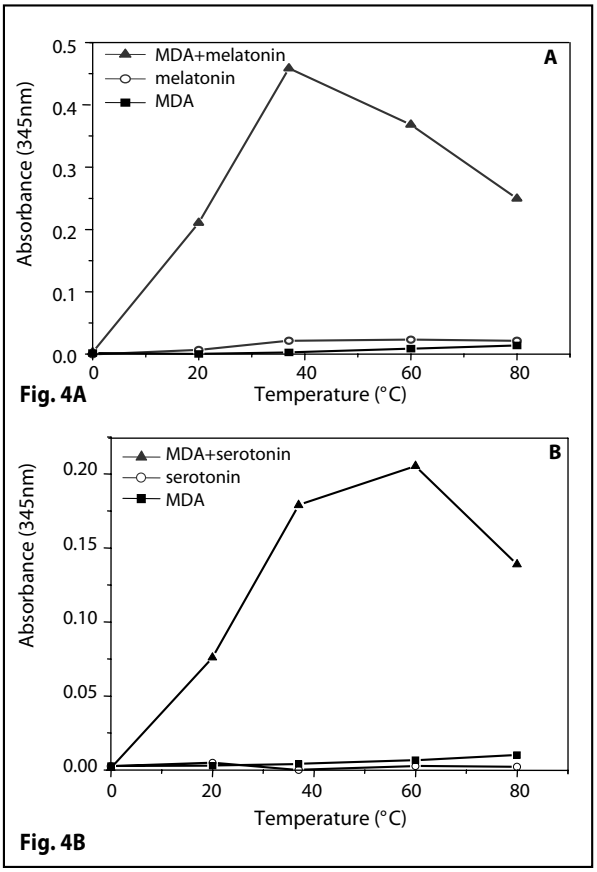

Figure 4 (A and B). Effect of temperature on the reaction of MDA with melatonin (A) or serotonin (B). 2mM MDA was incubated with 2mM melatonin or 2mM serotonin in water, pH 1.5 at the temperature of 0°C, 20°C, 37°C, 60°C, 80°C respectively. Absorbance at 345 nm was measured after 4h reaction. Data were a representative of several tests.

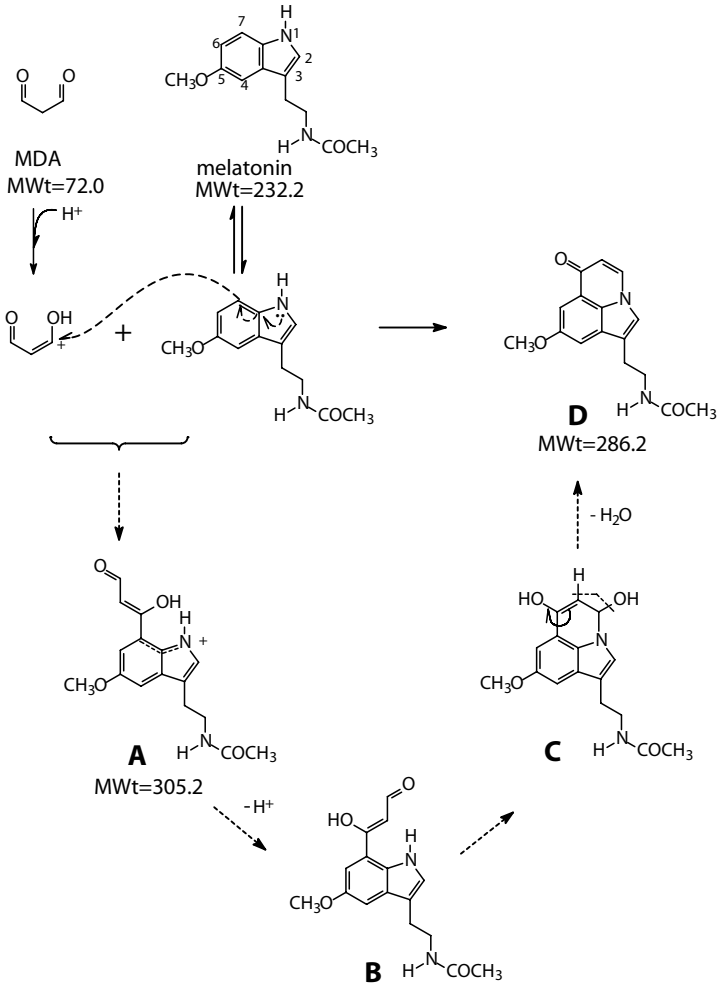

Figure 5 (A, B, C and D). Proposed structures of MDA+melatonin reaction. Adducts A, B and C were the proposed intermediates. Melatonin may react with a protonated MDA to form a product D in acidic condition.

chemistry (Fig. 5). An adduct (Fig. 5-D) with a molecular weight of 286.2 Dalton was found matching with a remarkable ion peak $[M_D+H]^+$ (m/z=287) in the full scan mass spectra. The LC/MS-ESI spectra are shown in Fig. 6.

Discussion

The pineal gland has an extraordinary capacity to integrate numerous hormonal and neural messages *via* several signal transduction pathways [9], and both melatonin and serotonin are important secretory products developed from it. Many biological glands' functions are found related with these two hormones.

In the past decades, melatonin has been shown to diminish oxidative damage, often detected by reducing MDA level, in both *in vivo* and *in vitro* experimental systems [13–15]. The function of melatonin was believed to scavenge the highly toxic hydroxyl radical, peroxynitrite anion, and possibly the peroxyl radicals. It may also stimulate mRNA levels for superoxide dismutase and the activities of glutathione peroxidase, thereby increasing its antioxidative capacity. The function of melatonin in keeping low thiobarbituric acid reactive substances (TBARS) levels during various oxidative stresses, in addition to its known beneficial effects on sleep, as well as its endocrine and immune functions [10, 13], brings about a very interesting question: Does melatonin help motivate physiological activities by reacting directly with toxic unsaturated carbonyls, which are a group of important biological garbages accumulated by various daily stresses [8, 18].

The amount of α,β-unsaturated carbonyls, e.g. best studied as MDA and 4-hydroxynonenal, is under an active control by different biological enzymes *in vivo* [19]. In mammals, a first defense against toxic unsaturated carbonyls takes place in the digestive system, where hepatic carbonyl dehydrogenases convert carbonyls to different compounds and eventually to carbon dioxide and water, or to soluble derivatives which can be excreted in the urine [4].

Although in healthy organisms reactions between carbonyls and amino compounds still occur, it is the reversibility of the primary reaction that keeps organisms' normality. When organisms produce relatively high concentrations of toxic carbonyls due to daily

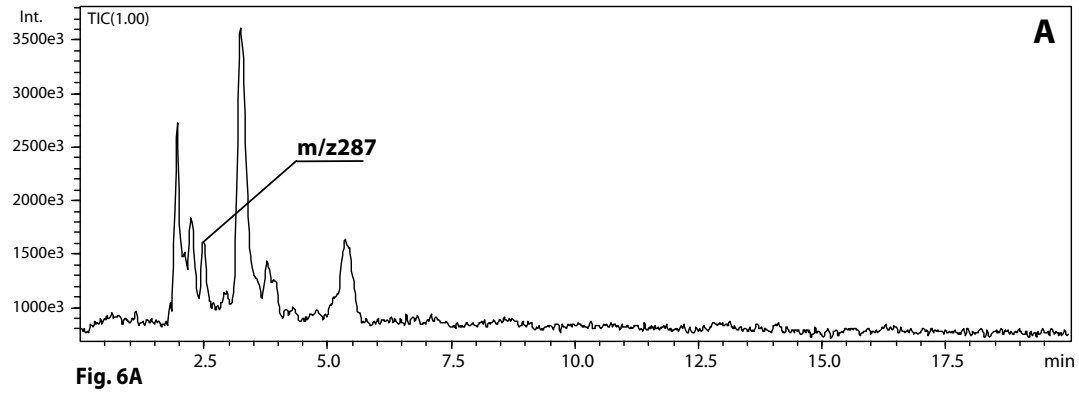

Fig. 6A

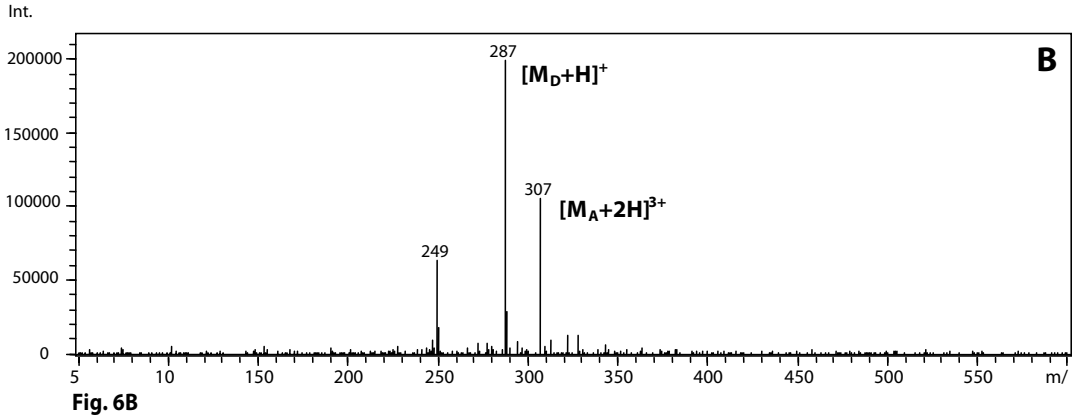

Fig. 6B

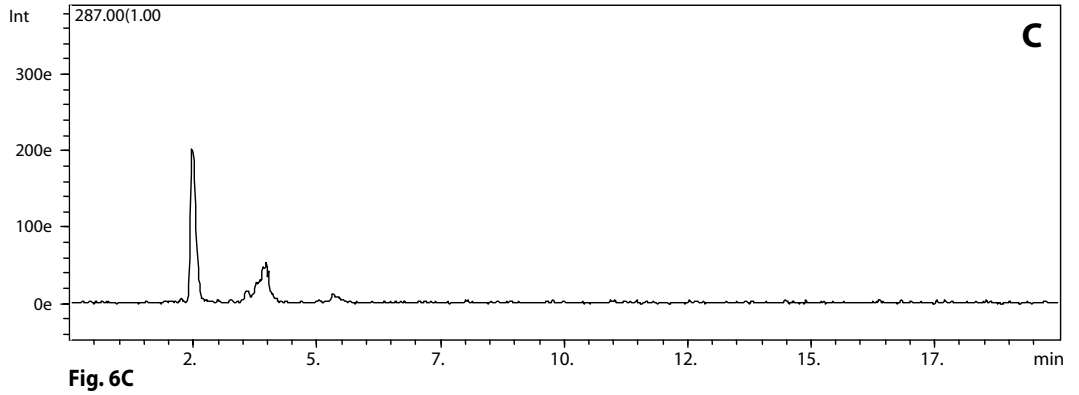

Fig. 6C

Figure 6 (A, B and C). Chromatograms by LC/MS-ESI positive ion scanning from m/z 50 to 600. (A): the total ion chromatogram, (B): the mass spectra corresponding to the total ion chromatogram at retention time 2.513min, (C): m/z 287.0 selective ion chromatogram. Reaction conditions were described in the section of material and methods.

activities, functional and structural proteins are likely to react with these substances to form mainly semi-bound protein-carbonyl compounds [4], biological defending substances, such as carbonyl dehydrogenases, glutathione and glutathione transferase [4, 20] all working intensively to reduce the toxic components and keep the carbonylation, (binding or further crosslinking) at a reasonably low level.

The recognition of a new reaction product demonstrated by absorption spectrum and LC/MS-ESI in this paper suggested an interesting function of melatonin in biological system. Although the new product has an absorption peak at 345nm, no relative fluorescence could be detected. The structure of the product can be a conjugated molecule formed by covalent inner-molecular crosslinking, where the third cyclic structure may establish the characteristics of the absorption at 345nm. As reported by Tan *et al* [21], a nitrogen molecule in the N-C=O structure being necessary for melatonin to form a new ring after melatonin interacted with a reactive species, and on the analogy of the formation process of TBA products [18], a possible structure of the MDA-melatonin adduct is proposed as shown in Figure 5D. Since the color of the product might fade at low pH, the chromatogen group of the product molecule was, therefore, suggested to be H^+ dependent. Considering that the reaction condition is basically non-physiologic, a more appropriate mechanism of melatonin's anti-carbonyl stress during sleep is under investigation.

It is interesting to note that, serotonin, an important neuronal transmitter, and the precursor of melatonin, can also react with MDA in the same system. This find-

ing may also provide a clue to the understanding of carbonyl stress in neuronal system.

In contrast to a paper from Allegra *et al* [22], which suggested that melatonin was not a substrate for MDA, our data demonstrated solid evidence showing a reaction possibility between these chemicals, particularly when an absorption increase of the new product with time and concentration was considered (Fig. 2 and 3). The cause of such difference between these two studies is not clear yet.

In summary, our results showed that the mechanism of the melatonin may diminish unsaturated carbonyls in one way or another. As carbonyls are important intermediates of oxidative stress, the results can be considered as a evidence for melatonin's special property of de-carbonylation. Furthermore, based on the carbonyl stress aging hypothesis [23], the results manifested that melatonin might play a significant role in antiaging process. Further studies are necessary to investigate a sleep-related de-carbonylation in brain and central nervous system.

Acknowledgements

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