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Measurement of Melatonin and its Metabolites

Importance for the Evaluation of Their Biological Roles

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Many physiologic changes related to light–dark cycles and antioxidant effects have been related to melatonin (*N*-acetyl-5-methoxytryptamine) and its metabolites, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK). In this review, we discuss some methodologies, in particular, those employing high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) assays to quantitatively determine melatonin, AFMK, and AMK. These approaches offer a highly specific and an accurate quantification of melatonin and its metabolites. These characteristics are essential to point out correctly the biological effects of these compounds in physiological and pathological conditions.

Key Words: Melatonin; N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK); mass spectrometry; labeled internal standard.

Biological Roles of Melatonin and of its Metabolites

Many physiological, endocrinological, and behavioral processes have been demonstrated to be mediated by melatonin (*N*-acetyl-5-methoxytryptamine). In higher vertebrates, melatonin is synthesized (mainly in the pineal gland and retina) at night under normal environmental conditions *(1)*. Among the functions of melatonin, the regulation of circadian rhythms is the best known *(2,3)*. Melatonin increases sleepiness, decreases core temperature, and increases peripheral temperature in humans *(4)*. Many of melatonin's regulatory roles are mediated through high-affinity and G protein–coupled receptors that reside primarily in the eye, kidney, gastrointestinal tract, blood vessels, and brain *(5)*.

Moreover, melatonin is known to exhibit antioxidant properties against the deleterious effects of reactive oxygen and nitrogen species (ROS and RNS, respectively) that are independent of its many receptor-mediated effects *(6–9)*. Melatonin possesses an electron-rich aromatic indole ring and acts as an electron donor, reducing and repairing eletrophilic radicals. It does not undergo redox cycling and can be considered a suicidal or terminal antioxidant *(8)*. Indeed, cyclic voltametry indicates that melatonin donates an electron at the potential of 715 mV. In this way, melatonin is irreversibly oxidized and cannot be recycled *(7)*. Melatonin has been reported to scavenge hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), nitric oxide ($NO^{\bullet}$), peroxynitrite anion ($ONOO^-$), hypochlorous acid (HOCl), singlet molecular oxygen [$O_2(^1\Delta_g)$] and superoxide anion ($O_2^{\bullet -}$) *(9)*.

Tan et al. *(10)* first demonstrated the ability of melatonin to scavenge the $^{\bullet}OH$. Mechanistic studies of melatonin interaction with ammonium 2,2"-azino-*bis*(3-ethylbenzthiazoline-6-sulfonate) (ABTS) cation radical provided evidence for a proposed mechanism that involves the formation of intermediates including the nitrogen-centered melatoninyl cation radical, the melatoninyl neutral radical, and cyclic 3-hydroxymelatonin (C3-OHM), which generates as final product N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) *(11)* (Fig. 1).

It was also reported that melatonin's ability to inhibit the auto-oxidation of lipids in homogeneous solution and in model heterogeneous systems is not due to its peroxyl radical trapping potential. In contrast, melatonin acts by inhibiting metal ion–catalyzed oxidation *(12)*. The interaction with another reactive species has also been reported *(9,13–15)*. The reaction of melatonin with H_2O_2 was dose-dependent *(13)* and leads to the oxidative cleavage of the indole ring to produce a compound identified as AFMK by electrospray ionization (ESI) mass spectrometry (MS), ^{1}H-NMR, and ^{13}C-NMR *(13,14)*. According to Tan et al. *(13)*, AFMK could be produced via a dioxetane intermediate, or through the standard alkene epoxidation reaction followed by hydrolysis to the diol, which is then oxidized to AFMK (Fig. 2).

Photosensitization of endogenous compounds and lipid peroxidation could generate $O_2(^1\Delta_g)$ in cells *(15)*. Because $O_2(^1\Delta_g)$ is very reactive toward electron-rich molecules,

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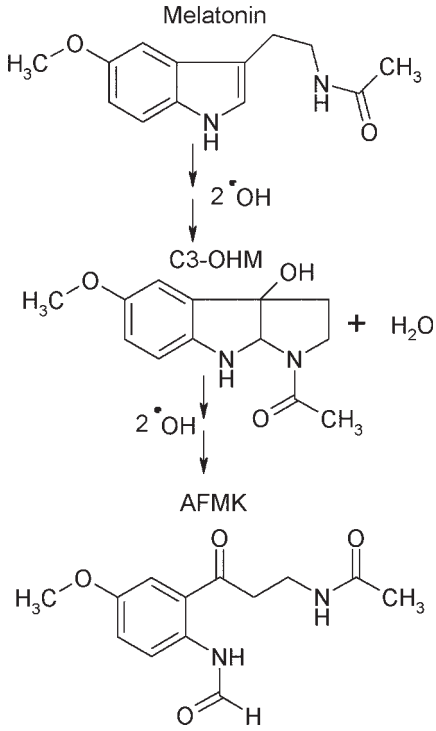

Fig. 1. Proposed mechanism for melatonin oxidation by •OH, which generates the cyclic intermediate C3-OHM. The latter product is further oxidized generating AFMK *(11,31)*.

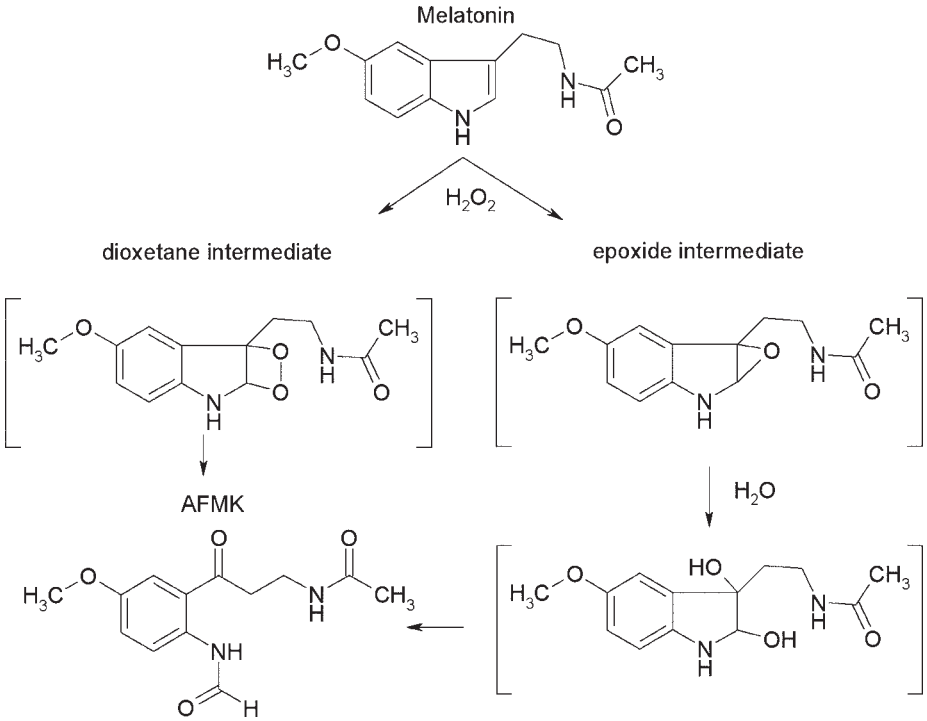

Fig. 2. Proposed mechanism for melatonin oxidation by H_2O_2. The H_2O_2 interaction with melatonin could occur through dioxetane intermediate or through the standard alkene epoxidation reaction followed by hydrolysis to the diol, which then is further oxidized to form AFMK *(13,14)*.

damage to biomolecules *(16)* may lead to many cytotoxic effects *(17)*. The formation of $O_2(^1\Delta_g)$ may involve different physical and chemical pathways (Fig. 3).

The major product of melatonin oxidation by $O_2(^1\Delta_g)$ was also identified as being AFMK *(18)*. In this study, melatonin was oxidized by using a clean chemical source of O_2

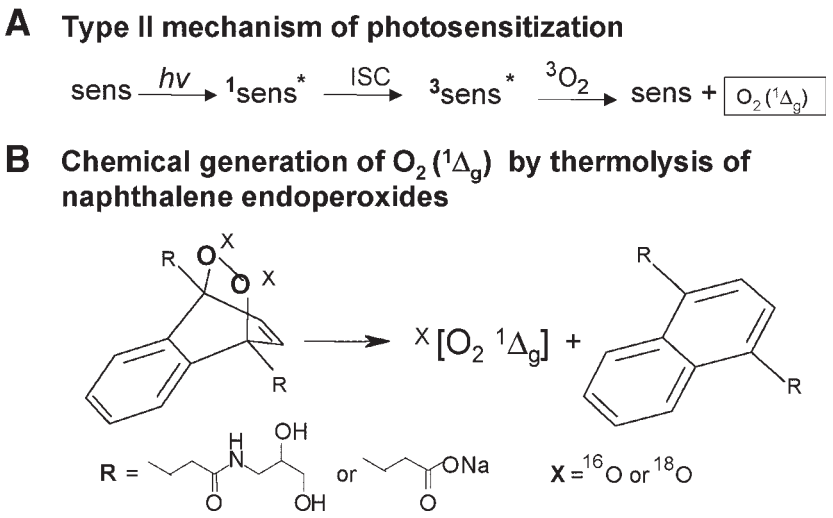

Fig. 3. Generation of $O_2(^1\Delta_g)$ by different sources. (**A**) Photosensitized process, type II mechanism generates $O_2(^1\Delta_g)$ by energy transfer. (**B**) Chemical source of $O_2(^1\Delta_g)$, thermolysis of naphthalene endoperoxides acting as a pure chemical source of $^{16}[O_2(^1\Delta_g)]$ or $^{18}[O_2(^1\Delta_g)]$ *(19)*.

($^1\Delta_g$), the thermodecomposition of the endoperoxide of *N,N'*-di(2,3-dihydroxypropyl)-1,4-naphtalenedipropanamide ($DHPNO_2$) and the resulting product were characterized by high-performance liquid chromatography coupled to ESI tandem MS (HPLC-ESI-MS/MS) and also by ^1H, ^{13}C, and dept135 NMR spectroscopy. An isotopically labeled $DHPN^{18}O_2$ was also used as a chemical source of labeled $^{18}[O_2(^1\Delta_g)]$ *(19)* to unequivocally characterize the product formed. The results confirmed the hypothesis that oxidation of melatonin by $O_2(^1\Delta_g)$ produces AFMK. The labeling and fragmenting pattern made possible the proposal of a mechanism involving a dioxetane intermediate (Fig. 4).

Moreover, it was also demonstrated that immune responsive cells could oxidize melatonin; this process involves myeloperoxidase and produces AFMK. This reaction consumes oxygen and exhibits chemiluminescence in the 440–540 nm region. Superoxide dismutase has a strong inhibitory effect on light emission but catalase and uric acid do not affect emission. An important role was proposed for this reaction in the inflammatory response, probably through a signaling process involving the generation of intermediate electronic excited species *(20,21)*.

To clarify if melatonin was a substrate of myeloperoxidase, the oxidation of melatonin by its redox intermediate compounds I and II was performed using transient-state spectral and kinetic measurements. The results revealed that both compound I and compound II oxidize melatonin via one-electron processes. Moreover, steady-state experiments showed that the rate of oxidation of melatonin was dependent on the H_2O_2 concentration *(22)*.

Melatonin mediates a variety of effects on mitochondrial functions. It has been shown that melatonin may physiologically regulate both the electron transport chain (ETC) and oxidative phosphorylation by increasing the electron

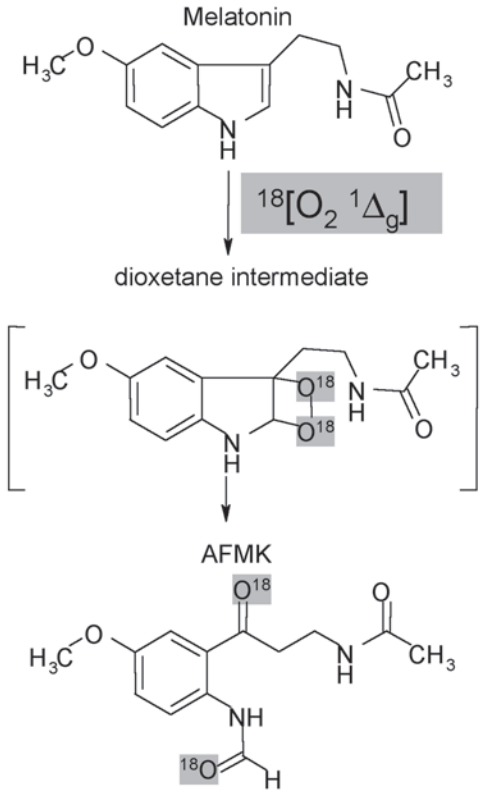

Fig. 4. Proposed mechanism for melatonin oxidation by $^{18}[O_2(^1\Delta_g)]$ generating ^{18}O-labeled AFMK *(18)*.

transport and ATP synthesis in normal mitochondria. Melatonin also counteracts mitochondrial oxidative damage induced by *t*-butyl hydroperoxide, recovering glutathione levels and ATP production. However, the effects of melatonin not only depend of its antioxidant properties, because the indoleamine specifically interacts with complex I and IV of the ETC increasing their activity *(23)*. It may rescue

mitochondria from oxidative stress–induced dysfunction and effectively prevent subsequent apoptotic events and death. Melatonin is able to prevent exogenously applied H_2O_2-induced mitochondrial swelling at an early time point and subsequently reduce apoptotic cell death. Melatonin also prevented mitochondrial ROS generation caused by other organic hydroperoxides including *t*-butyl hydroperoxide and cumene hydroperoxide *(24)*.

Interestingly, the antioxidant melatonin has also been shown to possess genomic actions, regulating the expression of several genes. Melatonin influences both antioxidant enzyme activity and cellular mRNA levels for these enzymes. The influence of melatonin on the activity and expression of the antioxidant enzymes glutathione peroxidase, superoxide dismutase, and catalase, both under physiological conditions and under conditions of elevated oxidative stress, reveals an additional role of this fascinating molecule *(25,26)*.

Physiological functions and in vivo levels of kynuramines, such as AFMK, are essentially unknown. Kynuramines and their metabolites are mainly produced by reactive oxygen species or by enzymes. Their presence in brain was first proposed by Hirata et al. *(27)*. AFMK has been found to counteract the deleterious effects of ROS/RNS in different systems *(28)*, suggesting this biogenic amine also plays an antioxidant role. In this respect, the cyclic voltammogram showed the presence of anodic waves that indicates the ability of the compound to donate its electrons. For AFMK, two anodic waves were detected at 456 and 668 mV, respectively, in the PBS buffer *(28)*. AFMK also protects macromolecules against oxidative damage formation induced by the combination of Cr^{3+} plus H_2O_2 and lipid peroxidation induced by Fe^{2+} plus H_2O_2 *(28)*.

In addition, the use of melatonin and AFMK as antioxidants in the treatment of oxidative stress induced by 5-aminolevulinic acid (ALA) was investigated *(29)*. It was shown that the compounds showed a dose-dependent protection of DNA damage induced by ALA/Fe^{2+} system in vitro, measured by plasmid DNA strand breaks and detection of 8-oxod Guo by HPLC coupled with electrochemical detection.

N^1-acetyl-5-methoxykynuramine (AMK), another metabolite deriving from melatonin by mechanisms involving reactive species, is formed by enzymatic or non-enzymatic deformylation of AFMK. It exhibits potent antioxidant properties exceeding those of its direct precursor AFMK. Contrary to AFMK, AMK was easily oxidized in a reaction mixture generating carbonate radicals *(30)*. Moreover, AMK proved to be highly protective in an oxidative protein destruction assay based on peroxyl radical formation. No prooxidant properties of AMK were detected. Thus, AMK may contribute to the antioxidant properties of the indolic precursor melatonin. Tan et al. *(8)* had already postulated that the continuous free-radical-scavenging potential of the original molecule (melatonin) and its metabolites may be defined as a scavenging cascade reaction.

Some other metabolites produced as a result of these scavenging actions of melatonin have been identified using pure chemical systems. The products AFMK and cyclic 3-hydroxymelatonin (C3-OHM) are formed when melatonin detoxifies hydroxyl radicals. Their abilities to protect DNA damage have been investigated *(31)*. They found that C3-OHM reduces $^{\bullet}OH$-mediated damage in a dose-dependent manner. In these studies, a hypothetical reaction pathway was proposed in which 1 mole of C3-OHM scavenges 2 moles of $^{\bullet}OH$ yielding AFMK as a final product (see Fig. 1).

Because melatonin and its metabolites are perceived as potential tools in the study of oxidative stress and the immune response in organisms, the development of sensitive and selective methods that allow for the precise detection of these compounds in tissues and fluids of animals is necessary. However, owing to its low concentration and the co-existence of many other endogenous compounds in blood, the determination of melatonin has been an analytical challenge.

This article discusses the current methodologies suggested in the literature for detection and quantification of melatonin and its metabolites and the relevance of their determination levels.

Determination of Melatonin, AFMK, and AMK in Biological Samples

In the past, some analytical methods had already been described to determine melatonin levels in biological samples, including bioassay and fluorimetry *(32,33)*. More recently, with new technologies available, reversed-phase HPLC with electrochemical or fluorimetric detection and gas chromatography coupled to mass spectrometry (GC-MS) have been applied to measure melatonin *(34)*.

Some other highly sensitive methodologies have also been reported *(35–40)* and a few fentomoles of melatonin have been detected. Nevertheless, in some cases *(35)*, it employs derivatization to generate fluorophore. This kind of approach lacks specificity, because other components from the biological sample may lead to the generation of fluorophores that may interfere with the correct level evaluation.

For monitoring of melatonin in biological fluids, use of immunological methods is the most widespread. Although the methods are highly sensitive and simple to use (lower limit of detection: 0.5 $pg \cdot mL^{-1}$), they inherently suffer from a potential risk of cross-reactivity to structurally similar compounds *(36)*.

A chemiluminescence (CL) method for the determination of melatonin has been reported. It is based on the observation that melatonin can greatly enhance the ultraweak CL between H_2O_2 and acetonitrile in alkaline solution. As a preliminary application, this simple method had successfully been applied to the determination of melatonin in health foods *(37)*. Singlet molecular oxygen was suggested to be produced by the reaction between H_2O_2 and acetonitrile

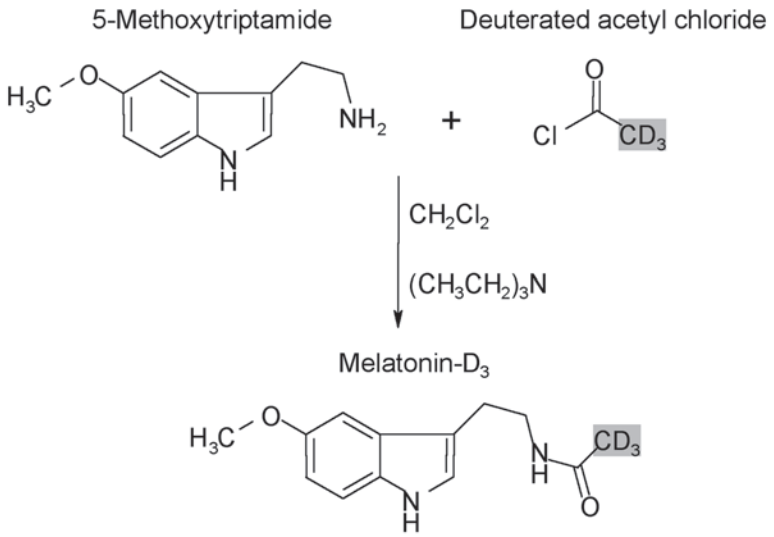

Fig. 5. Synthesis of labeled melatonin-D_3 through the reaction of 5-methoxytryptamine with deuterated acetyl chloride in dry dichloromethane, and in the presence of dry triethylamine *(43)*.

and responsible for the CL of melatonin. To prove this hypothesis, Almeida et al. *(38)* reported direct evidence on the formation of $O_2(^1\Delta_g)$ in the reaction of H_2O_2 with acetonitrile in alkaline solutions. The formation of $O_2(^1\Delta_g)$ was characterized by the dimol light emission in the red spectral region (>610 nm) using a red-sensitive photomultiplier; the monomol light emission in the near-infrared region (1270 nm) with a photomultiplier coupled to a monochromator; and the quenching effect of sodium azide. A direct spectral characterization of the near-infrared light emission attributed the emission to the transition of $O_2(^1\Delta_g)$ to the triplet ground state $O_2(^3\Sigma_g)$ was done to unequivocally demonstrate the presence of $O_2(^1\Delta_g)$.

The GC-MS technique is sensitive and offers more specificity; however, a difficulty is the need of derivatization *(39)*. Because of this, a valuable alternative is HPLC-ESI-MS/MS *(40–43)*. It has been considered very appropriate to investigate biological issues, because of many inconvenient steps of other methods are masked.

A limitation of this approach is the need of an internal standard. Yang and co-workers *(40)* reported a methodology by using acetyltryptamine as internal standard; however, this is not the ideal situation. The most appropriate is the employment of a labeled internal standard whose structure is the same as the analyte with a mass difference. The addition of an isotopically labeled internal standard prior to analysis improves the method's confidence level. Another analytical method enabled the determination of endogenous melatonin in human saliva, by using column-switching semi-microcolumn liquid chromatography/mass spectrometry and selected ion monitoring (SIM). Melatonin was monitored based on its fragment ion at *m/z* 174 by in-source dissociation and using deuterated melatonin (*N*-[2-(5-methoxy-1H-indol-3-yl)tetradeuteroethyl]trideuteroacetamide, 7-D-melatonin) as internal standard *(41)*. The main limitation of this methodology is the use of SIM mode to detect the ions generated in the probe, which does not imply an absolute specificity. Yet, using 7-D-melatonin as an internal standard, Eriksson et al. *(42)* reported a method for the determination of melatonin in human saliva using HPLC-MS/MS. The limit of detection was 1.05 $pg \cdot mL^{-1}$ and the limit of quantification was 3.0 $pg \cdot mL^{-1}$. This chromatographic method has been used to determine the circadian rhythm of melatonin among three nurses working the night shift and a patient suffering from an inability to fall asleep at night *(42)*.

Recently, Almeida et al. *(43)* have reported on the development of a new HPLC-ESI-MS/MS assay to quantitatively determine melatonin and AFMK. The stable isotopic internal standard melatonin-D_3 employed was easily synthesized by the reaction of 5-methoxytryptamine with deuterated acetyl chloride (CD_3COCl) (Fig. 5). Labeled AFMK (AFMK-D_3) was obtained after photo-oxidation of melatonin-D_3.

The predominant ion $[M+H]^+$ in the full scan mass spectra of melatonin, melatonin-D_3, AFMK, and AFMK-D_3 were located (Fig. 6). The fragments generated in collision-induced dissociation chamber revealed a predominant fragment at *m/z* 174 for melatonin and melatonin-D_3 (loss of the *N*-acetyl group), and at *m/z* 178 for AFMK and AFMK-D_3 (loss of both the *N*-acetyl and the *N*-formyl groups). The *m/z* transitions from 233 to 174 (melatonin), from 236 to 174 (melatonin-D_3), from 265 to 178 (AFMK), and from 268 to 178 (AFMK-D_3) were therefore chosen for the multiple reaction monitoring (MRM) detection experiments, which ensured a higher specificity and an accurate quantification of melatonin and AFMK in human plasma (Fig. 7). It must be added that AMK-D_3 internal standard was also obtained and its detection can be performed under the same

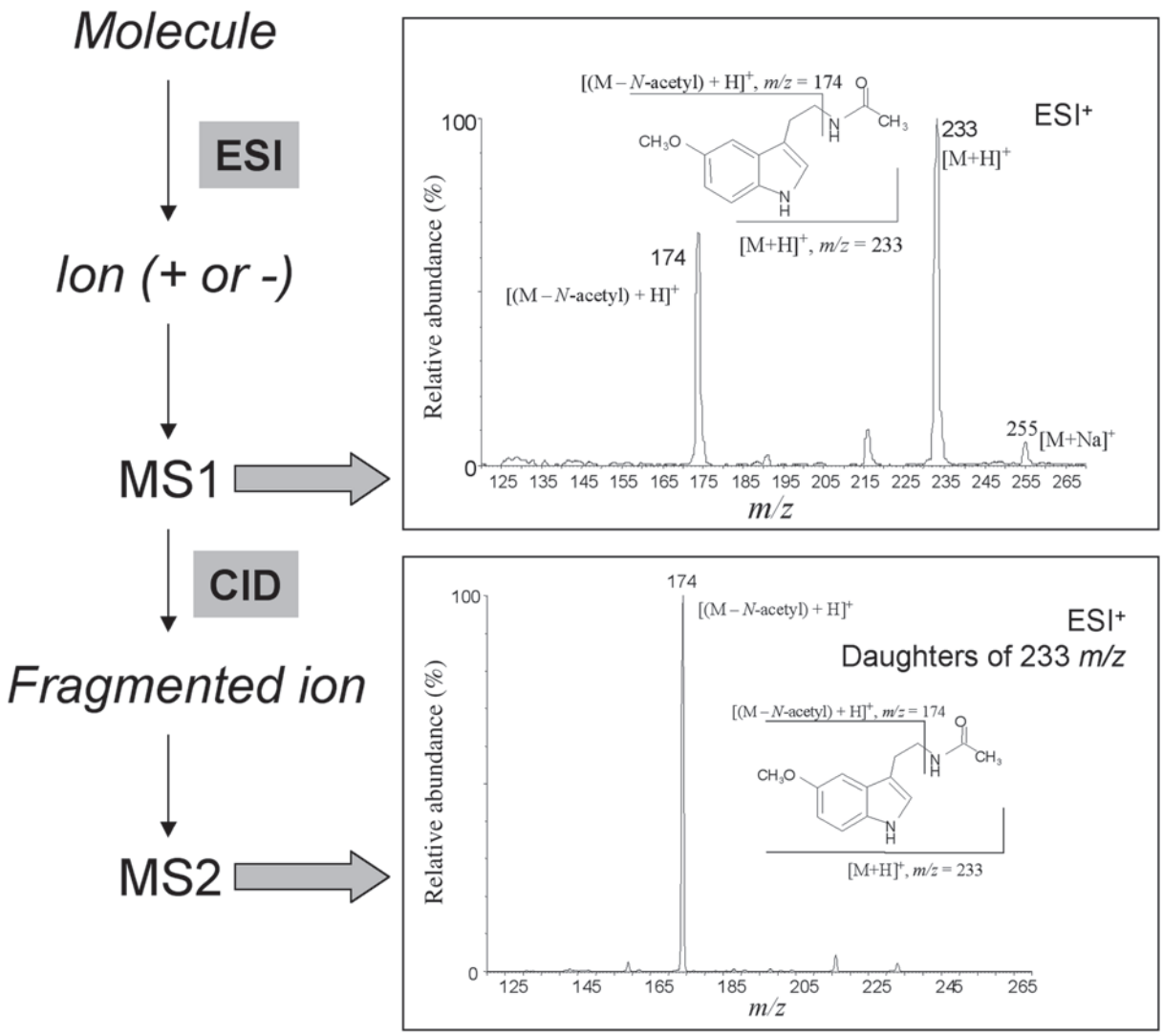

Fig. 6. The molecule is ionized by electrospray mode, the ion generated is detected in the first analyzer (MS1) and selected for fragmentation by collision-induced dissociation (CID), the fragmented ion is detected in the second analyzer (MS2). The spectra of melatonin in the MS1 and MS2 analyzers are shown *(43)*.

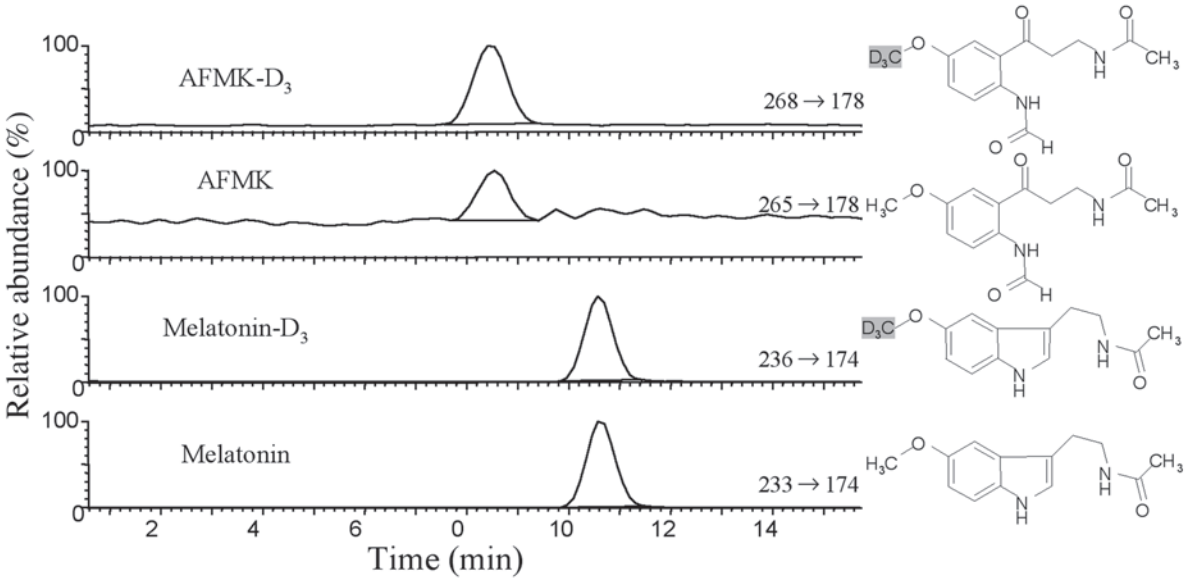

Fig. 7. Multiple reaction monitoring (MRM) detection, showing representatives' chromatograms of melatonin and AFMK and respective internal standards.

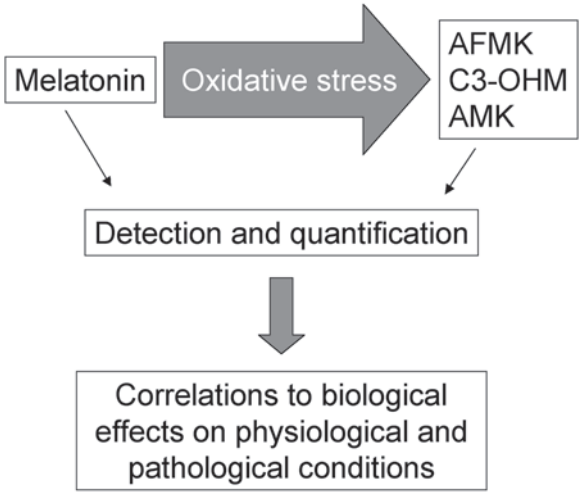

Fig. 8. Implications for the determination of melatonin and its metabolites levels.

conditions described above by selecting the corresponding ion transition (unpublished observations).

In addition, endogenous AFMK detection using HPLC with fluorescence detection in the retina of rats was reported. However, the serum concentration of this melatonin metabolite was below the detection limit of the measurement method. Retinal AFMK concentration was higher during the dark phase of the light/dark cycle, when the retinal melatonin content is maximal. Intraperitoneal administration of melatonin significantly increased serum and retinal AFMK levels *(44)*.

An HPLC-based method was used for AFMK and AMK determination in neutrophil and peripheral blood mononuclear cell culture supernatants. AFMK was detected by fluorescence (excitation 340 nm and emission 460 nm) and AMK by UV-VIS absorbance (254 nm) *(45)*.

Another methodology to detect AFMK previously reported, was based on radioimmunoassay *(46);* this assay does not show high sensitivity and presents some cross-reactivity of a range of indoles or kynuramines, that even at low concentration may lead to inaccurate results.

Concluding Remarks

Oxidative stress-induced mitochondrial dysfunction has been shown to play a crucial role in the pathogenesis of a wide range of diseases and melatonin has been shown to perform important effects on these systems. The efforts to develop and ameliorate methodologies for measurement of melatonin and AFMK concentrations are important, since their levels in various neurological pathologies such as Alzheimer or Parkinson's disease could contribute to construct a better correlation between oxidative stress and antioxidant defenses in these and other situations (Fig. 8).

Acknowledgments

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