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Radioimmunoassay of N-acetyl-N-formyl-5-methoxykynuramine (AFMK): a melatonin oxidative metabolite

Catherine Harthé[a], Danièle Claudy[b], Henri Déchaud[a], Berthe Vivien-Roels[c], Paul Pévet[c], Bruno Claustrat[a,*]

[a] *Service de Radioanalyse, Hôpital Neurocardiologique, Centre de Médecine Nucléaire, 59 boulevard Pinel, 69394 Lyon, Cedex 03, France*
[b] *Service d'Immunologie, Hôpital Neurologique, Lyon, France*
[c] *URA-CNRS 1332, Neurobiologie des Fonctions Rythmiques et Saisonnières, Université Louis-Pasteur, Strasbourg, France*

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Abstract

N-acetyl-N-formyl-5-methoxykynuramine (AFMK) is a melatonin metabolite identified in rat brain by Hirata et al. (The Journal of Biological Chemistry 249 (1974) 1311). Since no assay has been described for its routine measurement, we have developed and validated such a radioimmunoassay.

We synthesized AFMK and *N*-acetyl-5-methoxykynuramine (AMK), in order to produce anti-AFMK antibodies and to standardize the assay. The tracer [^{3}H]-AFMK was obtained from [^{3}H]-melatonin. The assay was preceded by a chromatographic step on Celite microcolumn in order to increase its specificity.

The assay was suitable for the measurement of AFMK levels ranging from 59 to 1894 pmol/L. The detection limit of the assay was routinely set at 65 pmol/L. The intra- and inter-assay coefficients of variation were 3.5% and 11% respectively. Investigation of the 24 h plasma pattern in healthy volunteers did not reveal any AFMK levels in plasma samples. In rats, plasma AFMK showed a peak after melatonin injection, which confirmed the in vivo AFMK production as a melatonin metabolite.

This AFMK assay is suitable for studies on melatonin metabolism.

Keywords: Melatonin; AFMK; AMK; RIA; Oxidative stress; Neurodegenerative disorders

* Corresponding author. Fax: +33-472357305.
E-mail address: bruno.claustrat@chu-lyon.fr (B. Claustrat).

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Introduction

Melatonin, which is the major indole compound synthesized by the pineal gland, displays a nycthemeral variation, as shown by high nocturnal but low or undetectable diurnal plasma concentration. The pineal gland plays an important role in maintaining homeostatic equilibrium in close relationship with changing environmental conditions. Melatonin is mainly metabolized by hydroxylation at the 6-position, then conjugated with sulfate and glucuronide. Although 6-sulfatoxy-melatonin is the major metabolite, Hirata et al. (1974), demonstrated in the rat that, besides the hepatic pathway, there is a cerebral oxidative metabolism leading to the conversion of melatonin to N-acetyl-N-formyl-5-methoxykynuramine (AFMK). Thereafter, AFMK is enzymatically converted to N-acetyl-5-methoxykynuramine (AMK), which can be detected in rat urine (Fig. 1). The discovery of melatonin as an antioxidant has opened up a new area of investigation. It is a potent scavenger of free radicals, particularly reactive oxygen species (ROS) such as hydroxyl radical, peroxyl radical and singlet oxygen (Tan et al., 1993). In both in-vitro and in-vivo experiments, melatonin has been shown to be effective in protecting cells against oxidative stress induced by ROS. Recently great interest has been given to the ROS involved in various human diseases, under the term of "oxidative stress". The hypothesis has been proposed that oxidative stress may be one pathway leading to

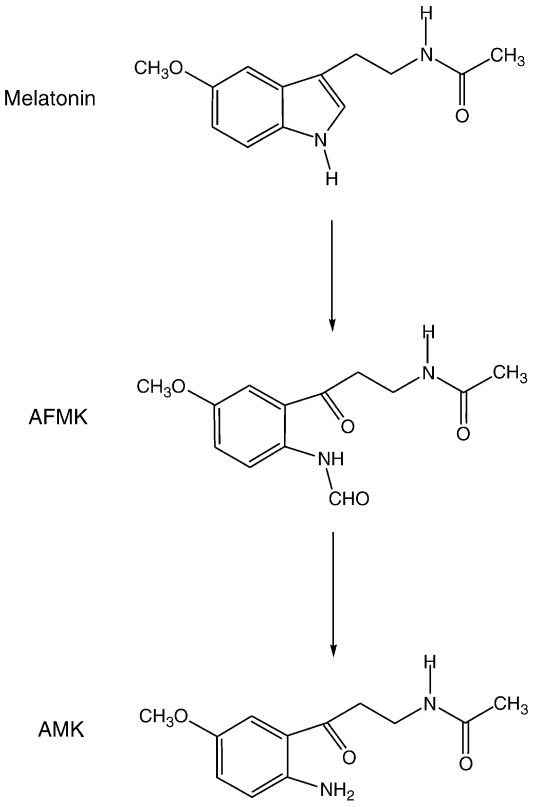

Fig. 1. Chemical structures and synthesis pathway for AFMK and AMK from melatonin.

neurodegenerative disorders such as dementia of Alzheimer type (Retz et al., 1998) and Parkinson disease (Fahn and Cohen, 1992; Foley and Riederer, 2000). Generally evaluation of oxidative stress is performed by measuring damaged biological products, and the choice of an indicator depends on the type of oxidative stress. Among the numerous indicators, only a few can be determined in clinical situations. Yet, the physiological function of AFMK remains unknown, in the absence of an available assay. We report here a first radioimmunoassay (RIA) for plasma AFMK using a tritiated tracer.

Materials and methods

Materials and chemicals

[O-methyl-^{3}H]melatonin, (TRK 798) was purchased from Amersham Pharmacia Biotech (Amersham, Bucks., UK). Protoporphyrin IX, melatonin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), albumin (bovine serum albumin BSA, fraction V) were from Sigma (St Louis, MO, USA), and 3-chloroperbenzoic acid from Fluka (Buchs, Switzerland).

Plates for thin-layer chromatography (TLC), Silica (G60,254) and silica gel 60 for column chromatography were obtained from Merck (Darmstadt, Germany), Celite was Primisil (125–200 μm) from BDH (Poole, England), Dextran T70 from Pharmacia Biotech AB (Uppsala, Sweden) and Ultima Gold MV from Packard Bioscience Company (Groningen, The Netherlands). Solvents and ordinary chemicals were of analytical grade from Merck. Phosphate buffer saline (PBS) consisted of 8 g NaCl, 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4 and 0.2 g of KCl per liter.

Mass spectrometry: the FAB mass spectra were obtained on a ZAB2-SEQ (Micromass Manchester, UK).

NMR spectrometry: Proton NMR spectra were recorded at 300 MHz on a Bruker AM 300 with $CDCl_3$ as solvent.

Reagent synthesis

Synthesis of AFMK by photolysis of melatonin

We first synthesized AFMK according to the method of Behrmann and Hardeland (1995). In the presence of protoporphyrin IX (PIX) as photocatalyst, melatonin was photooxidized by U.V. light and converted into AFMK.

In brief, 0.1 mL melatonin 2 mM and 0.1 mL PIX 0.1 mM in dimethylsulfoxide, was exposed in polycarbonate Petri dishes for 24 hours to U.V. light (13 lux, 360 nm). The course of the reaction was followed by silica gel TLC. The chromatographic system was: toluol/ethyl acetate/acetic acid (28/67/5). AFMK was visualized under U.V. light (254 nm or 360 nm), and eluted in methanol.

Synthesis of AFMK by chemical oxidation of melatonin

Alternatively, in order to produce a greater amount, AFMK was synthesized as described by Kennaway et al. (1988). In brief, melatonin (0.5 g, 2 mmol) in dichloromethane (30 mL) was stirred at 4 °C with 3-chloro-perbenzoic acid (2 g, 0.011 mol) for 18 h. The mixture was washed

with saturated aqueous sodium hydrogenocarbonate, dried, and evaporated to provide an orange oil. The oily residue was first purified by silica gel column chromatography with chloroform/isopropylalcohol (95/5). AFMK was located and identified using TLC with AFMK previously produced by photooxidation as reference. After separation on the silica gel column, the compound was isolated and purified again by preparative HPLC [λ = 254 nm, RP C18] with methanol/water (40/60).

Synthesis of AMK

AFMK (5 mg) was boiled in ethanol (1 mL) containing 125 μL 0.1 N HCl for 10 min. The reaction mixture was diluted with ethyl acetate and AMK was extracted with HCl 1.2 N. The extract was then purified by preparative HPLC (λ = 254 nm, RP C18), with methanol/water/acetic acid (40/60/1).

Synthesis of [O Methyl-^{3}H] AFMK and [O Methyl-^{3}H] AMK

9.25 MBq [O-Methyl-^{3}H] melatonin, specific activity of 3.07 TBq/mmol, and 0.1 mL PIX were dissolved in 0.5 mL DMSO, and exposed for 24 hours to U.V. light (13 lux, λ = 360 nm)). After removal of the solvent by directing a gentle stream of nitrogen, the residue was taken up in 50 μl methanol, and the chromatographic analysis was carried out by TLC, using silica gel coated plate, with toluol/ethyl acetate/acetic acid (28/67/5) as mobile phase, and followed by scanning. The peak, revealed by the radiochromatogram and comigrating with authentic AFMK (detected by U.V.), was eluted in methanol.

As described for the synthesis of AMK from AFMK, [^{3}H] AMK was prepared from [^{3}H] AFMK, and purified by TLC as above. The peak, which comigrated with authentic AMK (detected by U.V.) was eluted with methanol.

Synthesis of the immunogen

AMK was bound to carboxylic acid residues of BSA via the amine function by the carbodiimide method: to 2.5 mg AMK dissolved in distilled water, 14 mg BSA and 100 mg EDAC were added. The mixture was incubated at room temperature for two days, then dialysed against tap water for two days with two changes (4 liters each).

Production of antisera

Three New Zealand rabbits were immunized over 9 months by subcutaneous dorsal injection of immunogen, approximately 250 μg per animal in 2 mL saline/complete Freund's adjuvant (50/50). Booster injections were given by the same route at a six-week interval. Rabbits were bled from the ear vein to test blood samples 10 days after each injection.

Determination of the 1-octanol/PBS partition coefficient

To compare the polarity of AFMK and AMK with melatonin, the partition coefficient was determined with a shake-flask method. One mL of 1-octanol was vortexed with 1 mL PBS containing melatonin, AFMK or AMK. The quantification was made by HPLC as previously described, with an analytical column (LichroCART 125-4, Purospher® RP 18e 5 μm). The 1-octanol/PBS ratio was calculated from the peak height.

Chromatographic separation before RIA

A chromatographic step was developed to separate plasma AFMK from melatonin and AMK before the RIA. The separation was carried out using partition chromatography on Celite column according to the method described by Abraham et al. (1972). Disposable 5 mL glass pipets were used as support for the Celite, a small glass bead was added at the bottom of the pipet prior to packing. The Celite is prewetted with water (1 mL for 3 g of Celite). The Celite column (3.6 ml mark on the pipet) was filled with isooctane (3 ml). Experiments were performed with tritiated melatonin, AFMK and AMK to evaluate the power of resolution of the solvent system. The mobile phase consisted of a mixture of ethyl acetate in isooctane with increasing polarity, in a stepwise fashion.

Radioimmunoassay

Reagents

The assay buffer(pH = 7.5) consisted of 6.35 g of Na_2HPO_4 2 H_2O, 3.8 g of KH_2PO_4, 9 g of NaCl, 1 g of NaN_3, and 2 g of gelatin per liter. The charcoal suspension contained 2.7 g of activated charcoal Norit A, and 0.27 g of Dextran T70 per liter of assay buffer.

Concentrations of AFMK and AMK standard were determined from absorption coefficient in U.V., λ = 338 nm for AFMK, and λ = 377 nm for AMK (after confirmation of the purity of the products). Standard AFMK or AMK was dissolved in methanol to produce a stock solution of 757 nmol/L (200 ng/mL). When stored at -20 °C, AFMK solution was stable as long as six months. For each assay, the standard solution was diluted in assay buffer to provide standards over the range of 59 to 1894 pmol/L (15.6 to 500 pg/mL).

Assay protocol

Plasmas were thawed at room temperature, and samples (0.1–0.5 mL) were pipeted into extraction tubes. To determine the analytical recovery of AFMK, [^{3}H] AFMK (1000 cpm or 2000 dpm) was added and the final volume adjusted to 1 mL with assay buffer. After extraction with 5 mL dichloromethane and vortex, the organic phase was removed and the solvent evaporated. The dried residue was dissolved in 3 mL of isooctane and was transferred to the Celite column. Elution was carried out stepwise: the column was rinsed with 8 mL of ethyl acetate-isooctane (20–80), then the column was filled with 2 mL of a mixture ethyl acetate-isooctane (35–65) and AFMK was collected in one fraction with 5 mL of this mixture. The fraction was evaporated and redissolved in 0.5 mL of assay buffer. The recovery of the extraction and chromatographic step was calculated by counting the radioactivity of a 50 μL aliquot of redissolved fraction.

AFMK was quantified in duplicate samples by RIA as follows: 100 μL of [^{3}H] AFMK(10,000 cpm) were mixed with the sample (200 μL redissolved fraction) or 200 μL standard (11.7 to 379 fmol/tube) and 100 μL of antiserum (diluted 1000-fold). The final volume was brought for each sample to 500 μL with assay buffer, and incubated for 18 hours at 4 °C. Bound and free AFMK were separated by addition of 500 μL cold dextran-coated charcoal suspension. After incubation at 4 °C for 10 min, and centrifugation at 3000 g for 15 min at 4 °C, the radioactivity of the resulting supernatants, containing the [^{3}H]AFMK bound to antibody, was counted in 5 mL of Ultima Gold scintillant. B/B0 versus concentration of AFMK standard were plotted and the best

curve was drawn. B0 represents the fraction of [^{3}H]AFMK bound to antibody in the absence of AFMK standard, B is the fraction of [^{3}H]AFMK bound to antibody at a known AFMK concentration. The concentration of plasma AFMK was determined from this curve, taking into account the recovery.

Analytical performance

We evaluated the specificity of the RIA by determining the relative potency of the following compounds: AMK, melatonin, 6-sulfatoxymelatonin (aMT6s), 6-hydroxymelatonin, 5-methoxytryptamine, 5-methoxytryptophol, N-acetylserotonin, kynuramine, and 3-hydroxy-kynurenine. The relative potency was calculated by dividing the quantity of cross-reactants displacing 50% of the antibody-bound [^{3}H]AFMK by the quantity of AFMK producing 50% of displacement.

We evaluated also the specificity of this assay towards melatonin and AMK after extraction and chromatographic separation.

The dilution linearity was tested by dilution of a rat plasma sample displaying a high AFMK concentration. Six replicated standard curves were set up with two plasma controls to ascertain the inter-assay precision. Six replicated plasmas were assayed on the same day to ascertain the intra-assay precision. The detection limit was defined as the concentration giving a displacement of three standard deviations of counts from maximum binding.

Physiological validation

We assayed plasma AFMK concentration in blood samples of six healthy volunteers drawn every three hours over 24 hours. Plasma AFMK concentrations were also determined in rats after subcutaneous injection of melatonin: Adult male Wistar rats (280–320 g; Iffa Credo, L'Arbresle, France) were raised under a photoperiod schedule of 12 h. light/12 h. dark (lights 7.00–19.00 h) and were given water and food at libitum. The study was conducted in accordance with the guidelines approved for animal experimental procedures by the French Ethics Committee (decree 87–848). Sixty animals were divided in 2 groups which were subcutaneously injected with melatonin (100 μg/rat, n = 30) or vehicule (n = 30). Five rats of each group were sacrificed at 0 min, 7.5 min, 15 min, 30 min, 60 min and 120 min. Plasma was collected and stored frozen (− 20 °C) until AFMK and melatonin determinations. Plasma melatonin levels were determined by RIA according to Brun et al. (1984).

Results

Reagent synthesis

Synthesis of AFMK

Photooxidation of melatonin gave AFMK as only one reaction product (Rf = 0.23) with a good yield (> 60%). Chemical oxidation allowed the use of a greater quantity of melatonin than photooxidation. Side effects, however, such as colored oxidative molecules, were produced at the same time. Several runs on silica gel chromatography were necessary to obtain pure AFMK.

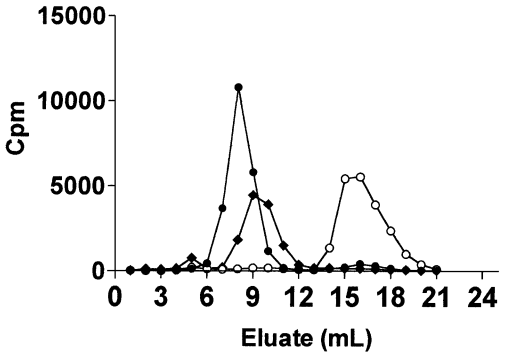

Fig. 2. Chromatographic profiles of melatonin (●), AMK (■) and AFMK (○) on Celite microcolumn. Solvent system, in a step fashion, is: 3 mL isooctane, 9 mL ethyl acetate in isooctane (20/80) and 9 mL ethyl acetate in isooctane (35/65).

Structural identification of crystallized AFMK was confirmed by NMR analyses and Mass spectrometry.

Synthesis of [O Methyl-^{3}H] AFMK

For the tracer synthesis, the radiochromatogram revealed a main peak comigrating with authentic AFMK, showing more than 80% of [^{3}H]-melatonin converted into [^{3}H]-AFMK. The concentrated tracer (0.74 MBq/mL) was stable for at least ten months without further purification.

Synthesis of AMK

When AFMK was boiled in ethanol containing HCl, AMK was obtained in 90% yield. Structural identification of crystallized AMK was confirmed by Mass spectrometry.

Synthesis of [O Methyl-^{3}H] AMK

Likewise, for the [^{3}H]AMK synthesis, the radio-chromatogram revealed one peak comigrating with authentic AMK.

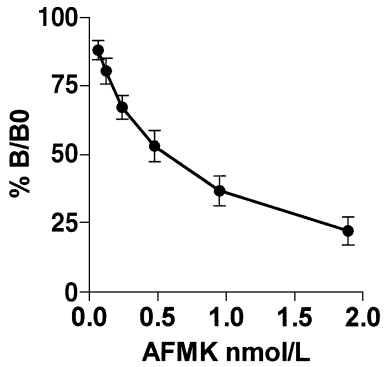

Fig. 3. Composite standard curve of AFMK, derived from six separate curves. Points are the mean ± SD.

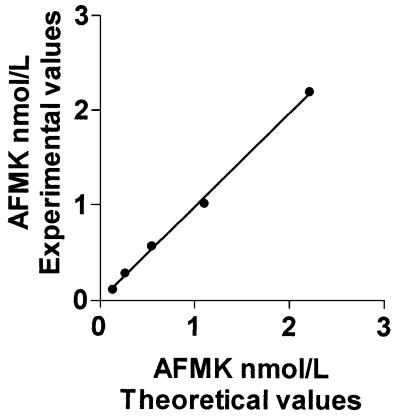

Fig. 4. Dilution linearity profile generated by a rat plasma with high AFMK concentration Y = 0.99x − 0.01, R^2 = 0.99.

Octanol/PBS partition coefficient

The partition coefficients P were P = 3 for AFMK, P = 5.5 for AMK and P = 14 for melatonin, this latter value being in concordance with that obtained by Pardridge and Mietus (1980).

Chromatographic separation

Melatonin and AMK were eluted on Celite microcolumn with 20% ethyl acetate in isooctane, and AFMK with 35% ethyl acetate in isooctane, according with their respective polarity (Fig. 2).

Radioimmunoassay

All the rabbits immunized produced antisera. The antiserum showing the highest titer was chosen for the RIA assay, with a 1/5000 final dilution. The affinity constant was 1.4 10^9 L/mol, calculated by the method of Scatchard (1949). The intra- and inter-assay coefficients of variation (CV) were respectively 3.5% and 11% at 700 pmol/L, and 568 pmol/L of AFMK was necessary for a 50% displacement of the

Table 1
Cross-reactivity data for AFMK RIA

Compound	% cross-reactivity
AFMK	100
AMK	100
a-MT6s	4
Melatonin	2
N-acetylserotonin	0.7
6-OH melatonin	0.2
5-Methoxytryptophol	0.04
5-Methoxytryptamine	0.004
3-Hydroxykynurenine	< 0.001
Kynuramine	< 0.001

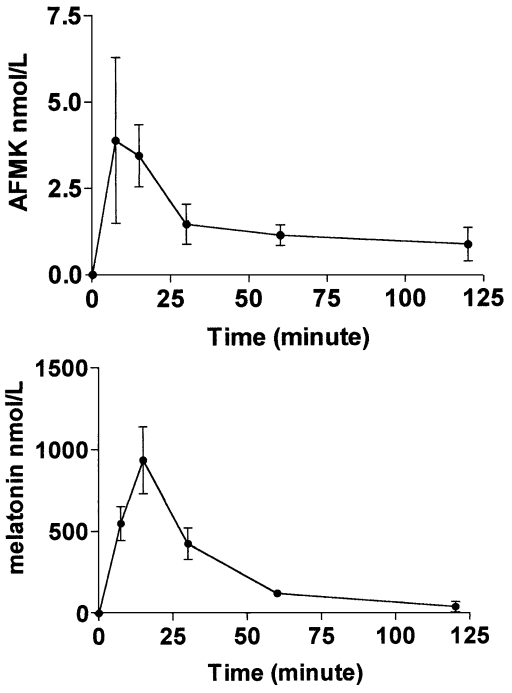

Fig. 5. Plasma AFMK and melatonin profiles observed in rats after melatonin injection. Points are the mean ± SD.

tracer (Fig. 3). The lowest detectable concentration of AFMK was 65 pmol/L. The dilution linearity is shown in Fig. 4. The percentage of cross-reactivity of the tested compounds was low, except for AMK (Table 1).

After extraction which discarded aMT6s and chromatographic separation, reactivities of melatonin and AMK were respectively 0.15% and 6 %. The AFMK recovery from plasma sample after extraction and Celite column chromatography was 60 ± 12% (n = 20).

Physiological validation

In healthy volunteers, plasma AFMK remained constantly below 65 pmol/L, even for nocturnal samples. On the other hand, after injection of 100 μg of melatonin in the rat, plasma AFMK increased from 7.5 min (maximum) then decreased and remained above the detection limit 2 h later. Melatonin concentration was maximum at 15 min (Fig. 5).

Discussion

Here we report on the first radioimmunoassay for the measurement of AFMK and detail its technical validation. AFMK and AMK were synthesized according to previously described methods. The photooxidation reaction of melatonin with a U.V. lamp was optimized in order to easily produce little amounts of AFMK with high yield. This procedure also allowed us to produce [^{3}H]AFMK from labelled

melatonin, a tracer showing a configuration similar to the hapten, and useful for recovery correction. It could be conserved for several months in methanol at −20 °C without degradation. The obtained antiserum was specific for both AFMK and AMK, as shown by the low percentage cross-reactivity of a range of indoles or kynuramines. The greatest cross-reactivities were observed with aMT6s (4%) and melatonin (2%), which could alter plasma AFMK levels in melatonin treated subjects. But, in this case, the solvant extraction discarded aMT6s and the chromatographic step which separates AFMK from melatonin improved the specificity of the assay. Similar reactivity of AMK in our immunoassay would be of interest to determine this compound in biological fluids, urine for example, upon suitable conditions. The separation of AFMK with melatonin and AMK by partition chromatography was facilitated by the great difference of polarity between these compounds, according to the P values.

Our RIA was reproducible and showed a good correlation between diluted plasmas and the AFMK standard curve. Nevertheless, the three rabbits produced low-titer antisera of poor affinity, which is not surprising considering the AFMK structure which includes only an aromatic ring and a linear chain. The detection limit set at 65 pmol/L was not sufficient for the detection of AFMK in plasma of healthy subjects, whatever the time of sampling. Since only a little amount of melatonin (< 10%) crosses the blood-brain barrier, a very little amount is converted into AFMK, of which the passage from cerebral fluid to blood is not known. Thus, it is not surprising that no AFMK was detected in human plasma. The evidence for the presence of AFMK was found only in the plasma of rats after melatonin injection, which confirmed the in-vivo AFMK production as a melatonin metabolite. In this case, the rapid increase in plasma AFMK levels suggests a conversion of melatonin to AFMK, via hepatic tryptophan 2,3-dioxygenase (Rodden and Berg, 1974).

Further studies are needed to improve the performances of the assay, especially sensitivity by increasing sample volume, and to elucidate the factors affecting AFMK levels. One factor may be oxidative stress because previous works have shown the antioxidant role of melatonin. It would be of interest to determine melatonin and AFMK concentrations in various neurological pathologies such as dementia of Alzheimer type, or Parkinson's disease where the role of oxidative stress has been considered (Cohen, 2000; Bowling and Beal, 1995). For Tan et al. (2001), AFMK might represent a valuable new antioxidant for preventing and treating free radical relative disorders. Our assay might be a reliable tool to monitor the evolution of oxidative stress and to evaluate efficiency of treatment. Meanwhile, this RIA will be useful to measure the AFMK production after melatonin ingestion in order to gain further insight into melatonin metabolism.

References

Abraham, G.E., Buster, J.E., Lucas, L.A., Corrales, P.C., Teller, R.C., 1972. Chromatographic separation of steroid hormones for use in radioimmunoassay. Analytical Letters 5, 509–517.

Behrmann, G., Hardeland, R., 1995. Protoporphyrin-mediated photooxidation of melatonin and 5-methoxytryptophol exposed to UV. In: Hardeland (Ed.), Cellular rhythms and indoleamines. Univ of Göttingen, pp. 135–139.

Bowling, A.C., Beal, M.F., 1995. Bioenergetic and oxidative stress in neurodegenerative diseases. Life Sciences 56, 1151–1171.

Brun, J., Claustrat, B., Harthe, C., Vitte, P.A., Cohen, R., Chazot, G., 1984. Melatonin RIA: analytical and physiological criteria of validity. Advances in the Biosciences, vol. 53, pp. 41–45.

Cohen, G., 2000. Oxidative stress, mitochondrial respiration, and Parkinson's disease. Annals of the New York Academy of Sciences 899, 112–120.

Fahn, S., Cohen, G., 1992. The oxidant stress hypothesis in Parkinson's disease: evidence supporting it. Annals of Neurology 32, 804–812.

Foley, P., Riederer, P., 2000. Influence of neurotoxins and oxidative stress on the onset and progression of Parkinson's disease. Journal of Neurology 247, 1182–1194.

Hirata, F., Hayaishi, O., Tokuyama, T., Senoh, S., 1974. In vitro and in vivo formation of two new metabolites of melatonin. The Journal of Biological Chemistry 249, 1311–1313.

Kennaway, D.J., Hugel, H.M., Clarke, S., Tjandra, A., Johnson, D.W., Royles, P., 1988. Structure-activity studies of melatonin analogues in prepubertal male rats. Australian Journal of Biological Sciences 41, 393–400.

Pardridge, W.M., Mietus, L.J., 1980. Transport of albumin-bound melatonin through the blood-brain barrier. Journal of Neurochemistry 34, 1761–1763.

Retz, W., Gsell, W., Munch, G., Rösler, M., Riederer, P., 1998. Free radicals in Alzheimer's disease. Journal of Neural Transmission 54, 221–236.

Rodden, F.A., Berg, C.P., 1974. Enzymatic conversion of L- and D-tryptophan to kynurenine by rat liver. The Journal of Nutrition 104, 227–238.

Scatchard, G., 1949. The attraction of proteins for small molecules and ions. Annals of the New York Academy of Sciences 51, 660–672.

Tan, D.X., Chen, L.D., Poeggeler, B., Manchester, L.C., Reiter, R.J., 1993. Melatonin: a potent, endogenous hydroxyl radical scavenger. Endocrine Journal 1, 57–60.

Tan, D.X., Manchester, L.C, Buckhardt, S., Sainz, R.M., Mayo, J.C., Kohen, R., Shohami, E., Huo, Y.S., Hardeland, R., Reiter, R.J., 2001. N1-acetyl-N2-formyl-5-methoxykynuramine, a biogenic amine and melatonin metabolite, function as a potent antioxidant. The FASEB Journal 15, 2294–2296.