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In Vivo Microdialysis/Liquid Chromatography/ Tandem Mass Spectrometry for the On-line Monitoring of Melatonin in Rat

Philip S. H. Wong, K. Yoshioka, F. Xie and P. T. Kissinger*
Bioanalytical Systems, Inc., West Lafayette, IN 47906, USA

Liquid chromatography/tandem mass spectrometry (LC/MS/MS) has been coupled to *in vivo* microdialysis for on-line monitoring of melatonin in a freely moving rat for a period of 15 hours. A microdialysis probe was surgically implanted into the jugular vein of the rat, and deionized water was used as the perfusion medium at a flow rate of 1.0 μL/min. Microdialysis samples were collected in an on-line injector with sample injection every 30 minutes. Melatonin was dosed by intraperitoneal (i.p.) injection and then monitored by microdialysis/LC/MS/MS. The whole experiment, including the microdialysis sampling and sample injection into the LC/MS system, was fully automated. Metabolites of melatonin were identified off-line by LC/MS^n experiments. Two metabolites were identified as 6-hydroxymelatonin and cyclic 2-hydroxymelatonin, consistent with ones found previously in the literature.

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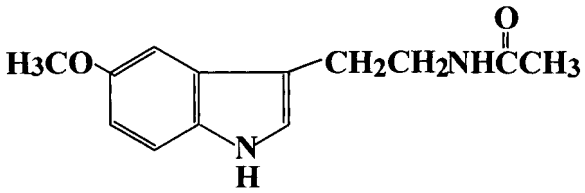
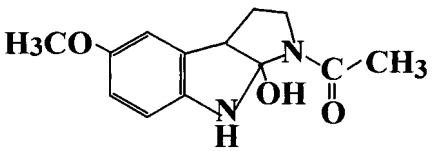
In recent years, microdialysis has gained wide recognition as an important technique for *in vivo* physiological and pharmacological studies especially in the area of drug distribution and metabolism.[1,2] *In vivo* microdialysis is a sampling technique involving a semipermeable membrane probe implanted in the tissue of interest, and the studies can be conducted in awake freely moving animals. During microdialysis, a perfusion medium is pumped through the membrane at low flow rates (typically 0.5–2.0 μL/min). The microdialysis membrane excludes large molecules (e.g. protein) and therefore no sample preparation is required. Collection times of about 5–30 minutes are usually necessary to obtain sufficient volume for analysis. To analyze these dialysates, a highly sensitive and selective detection method is needed. Liquid chromatography/mass spectrometry (LC/MS) with atmospheric pressure ionization (API) provides a powerful tool for the rapid and sensitive determination of a wide variety of organic compounds in complex biological matrices.[3–5] Recently, microdialysis with mass spectrometric detection has emerged as an important tool in biochemical research.[6–10]

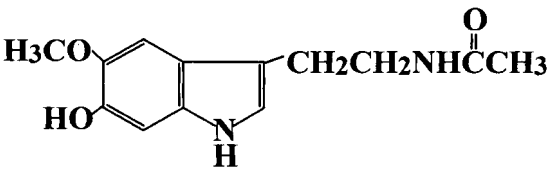
Melatonin (*N*-acetyl-5-methoxytryptamine, Structure I) is one of the neurochemically important indole compounds and has been used as a non-regulated therapeutic agent against jet-lag[11] and sleep disorder.[12] It has been suggested that melatonin may delay the aging process.[13,14] We have previously developed a simple and reliable method for the determination of melatonin by liquid chromatography/ electrochemistry (LCEC) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) with atmospheric pressure chemical ionization (APCI).[15] The present paper demonstrates the application of on-line coupling of *in vivo* microdialysis with LC/MS/MS for the study of the metabolism of melatonin in a freely moving animal.



Structure 1. Melatonin



Structure 2. Cyclic 2-hydroxymelatonin



Structure 3. 6-hydroxymelatonin

EXPERIMENTAL

(1) *In vivo* microdialysis

Microdialysis sampling was performed using a BAS syringe pump (MD-1001) coupled to a 10 mm vascular microdialysis probe (MD-2310) which was surgically implanted in the jugular vein of a Sprague-Dawley male rat (300–350g). The probe was perfused with deionized water at a flow rate of 1.0 μL/min. Water was used as the perfusion medium in order to reduce the salt content in the APCI source as high salt content decreases the sensitivity.

*Correspondence to: P. T. Kissinger, Bioanalytical Systems, Inc., West Lafayette, IN 47906, USA.

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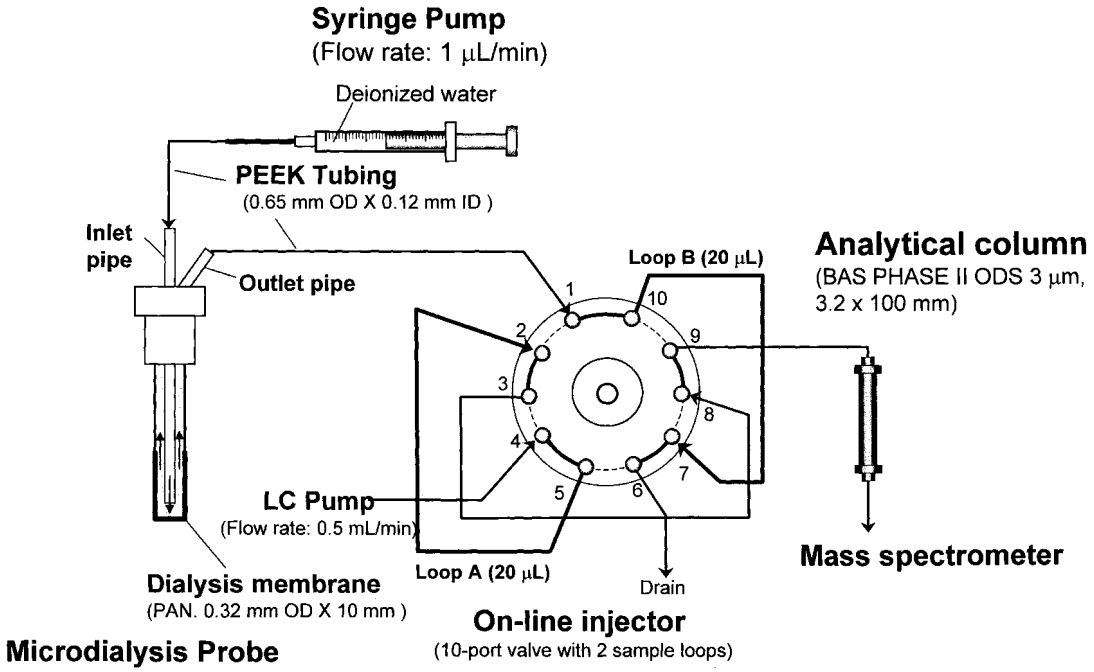

Figure 1. Schematic diagram of the experimental setup for the on-line microdialysis/LC/MS/MS experiment.

Alternatively, this problem can be totally avoided by using a divert valve after the LC column, before the inlet of the mass spectrometer. Concentration of melatonin in the dialysate was monitored by on-line LC/MS/MS with a BAS Pollen-8 on-line injector (20 μL loop) with sample injection every 30 minutes for a period of 15 hours. During microdialysis sampling, the awake unrestrained rat was housed in the BAS "Beekeeper Rodent Residence". The relative *in vitro* recovery was determined by placing the microdialysis probe into a vial containing a 1 mL aliquot of 50 ng/mL melatonin. The relative recovery ($\text{recovery}_{\text{in vitro}} = C_{out}/C_i$) was calculated by comparing the areas under the peaks in the chromatograms using selected reaction monitoring (233 → 174) for melatonin in the dialysate (C_{out}) and in the medium (C_i).

(2) Melatonin administration

10 mg of melatonin (Research Biochemicals International, MA, USA) were dissolved in 1 mL of 10% ethanol, and 0.3 mL of this solution was dosed into the rat by intraperitoneal (i.p.) injection.

(3) LC/MS system

The LC/MS system consisted of a Quattro LC triple quadrupole mass spectrometer (Micromass, UK) equipped with an atmospheric pressure chemical ionization (APCI) source. The analytical column was a Biophase II C_{18} column, 3 μm, 100 mm × 3.2 mm (Bioanalytical Systems, Inc., West Lafayette, IN,USA) and the mobile phase was 30% acetonitrile and 1% acetic acid in 10 mM ammonium acetate solution. The mobile phase was set at a flow rate of 0.5 mL/min driven by a PM-80 pump (BAS). The mass spectrometer was operated in APCI positive ion mode. Selected reaction monitoring (SRM) of the parent ion *m/z* 233 and the product ion *m/z* 174 was used to monitor the concentration of melatonin. A collision energy of 16 eV, at an argon gas pressure of 2 mTorr (multiple collision conditions) was used. Metabolites of melatonin were identified by MS^n experiments performed using a Finnigan LCQ ion trap mass spectrometer with a mobile phase of 10% acetonitrile in 10 mM ammonium acetate and 1% acetic acid at a flow rate of 1.0 mL/min.

RESULTS AND DISCUSSION

The experimental setup for on-line microdialysis/LC/MS/MS is shown schematically in Fig. 1. The retention time of melatonin was 2.8 minutes. A calibration curve was constructed by using external standard solutions. Aqueous standard solutions of melatonin were infused into the 20 μL loop of the on-line injector without passing through the microdialysis probe. The areas of the chromatograms were computed in order to construct a calibration curve. Figure 2 shows the calibration curve with a dynamic range of 3 orders of magnitude. The detection limit is about 10 pg on column, which is much lower than that we reported previously (50 pg) using an ion trap mass spectrometer.[15]

Before the administration of melatonin to the rat, three dialysate control samples were collected and injected on-line into the LC/MS system. Figure 3 shows the concentration-time profile obtained, and it is observed that melatonin reached a maximum concentration (~800 ng/mL) about one hour after administration, and decreased to almost zero after about 4 hours. The significance of this experiment is that it requires minimum labor, and the whole experiment is automated. Also, the sampling time can be shortened (e.g. to 10 min) in order to provide better time-resolved data. This is programmable with the Pollen-8 injector.

Generally, the recovery of free analyte from sample by microdialysis is a limitation of this methodology in pharmacokinetic studies. The relative *in vitro* recovery was found to be about 53%. This high recovery is due to the longer probe (10 mm). Relative recoveries from small membrane probes are in the 1 –20% range. In this

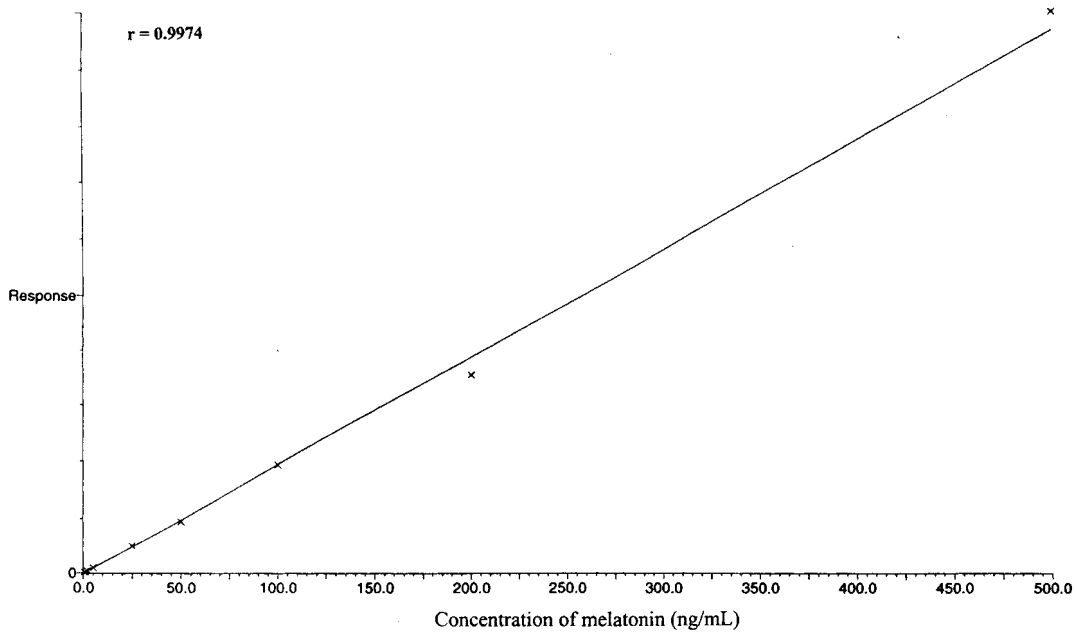

Figure 2. LC/MS/MS calibration curve for melatonin (*m/z* 233 → 174).

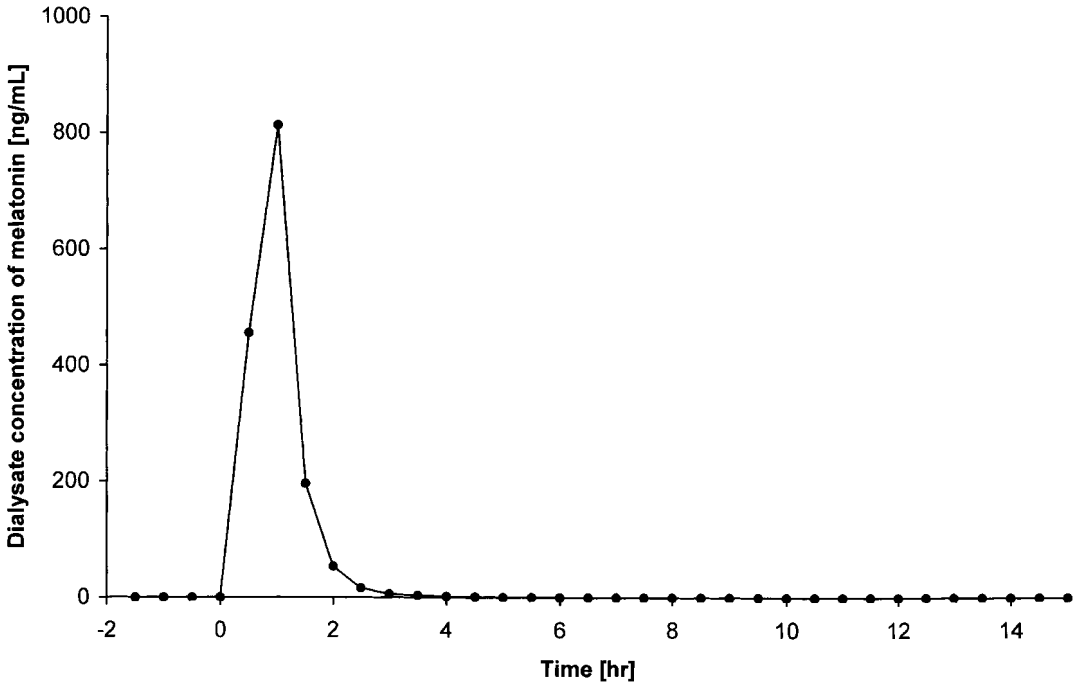

Figure 3. Concentration-time profile of melatonin for a period of 15 hours following i.p. administration.

experiment, it is desirable to maximize the recovery as the concentration of the analyte is low. However, if endogenous substances with sensitive feedback mechanisms are studied, removing significant amounts could alter the physiological parameters and yield artificial data. In that case, recovery should be minimized.

We have found that when melatonin (*m/z* 233) undergoes chemical oxidation (using H_2O_2 as the oxidizing agent), it forms mono-oxidation (*m/z* 249) and di-oxidation products (*m/z* 265),[16] which are 16 and 32 Da heavier than melatonin (addition of one and two oxygen atoms, respectively). So, we suspect these products may be formed during melatonin metabolism. Dialysate samples were collected from 1–4 hours of microdialysis. The samples were then analyzed off-line by LC/MS^n experiments with a Finnigan LCQ ion trap mass spectrometer. Three target compounds (melatonin, mono-oxidation and di-oxidation products) were analyzed by LC/MS/MS. From 0–7 min, a MS/MS scan function (249 → products) was performed in order to monitor the mono-oxidation product (*m/z* 249). From 7–11 min, another scan function (265 → products) was used to monitor the di-oxidation product. From 11–18 min, melatonin was moni-

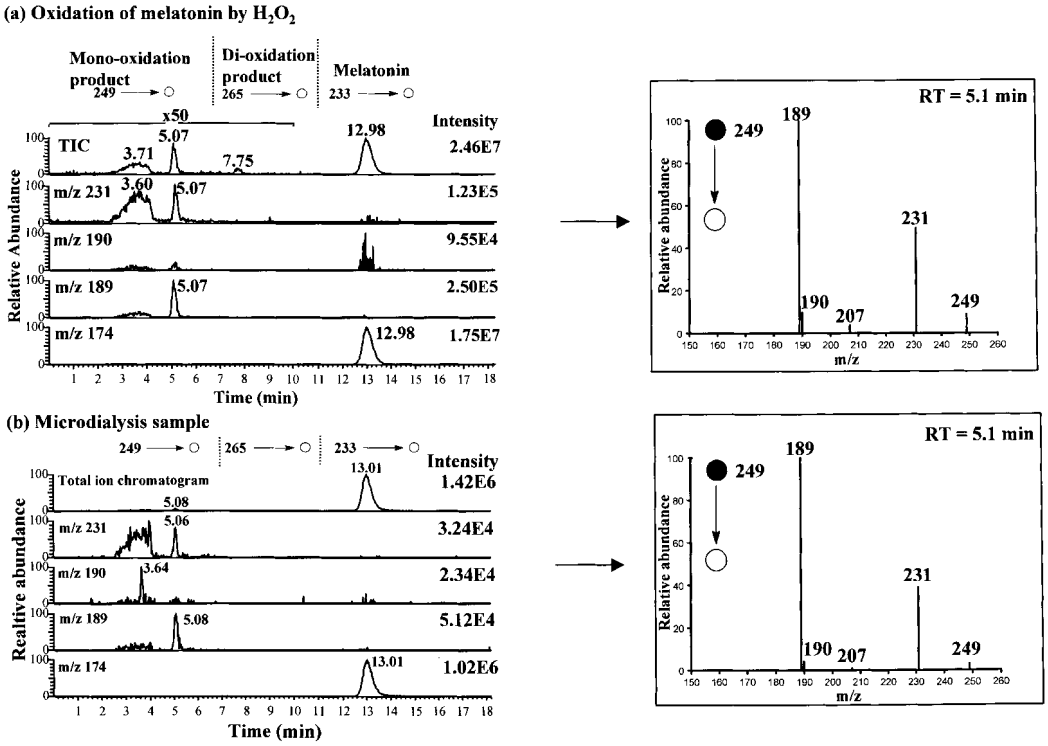

Figure 4. Product ion scans of (a) oxidation products of melatonin by H_2O_2 and (b) microdialysis sample. The matches of retention time (5.1 min) and MS/MS spectra confirm that the peak eluted at 5.1 min is the mono-oxidation product of melatonin (cyclic 2-hydroxymelatonin).

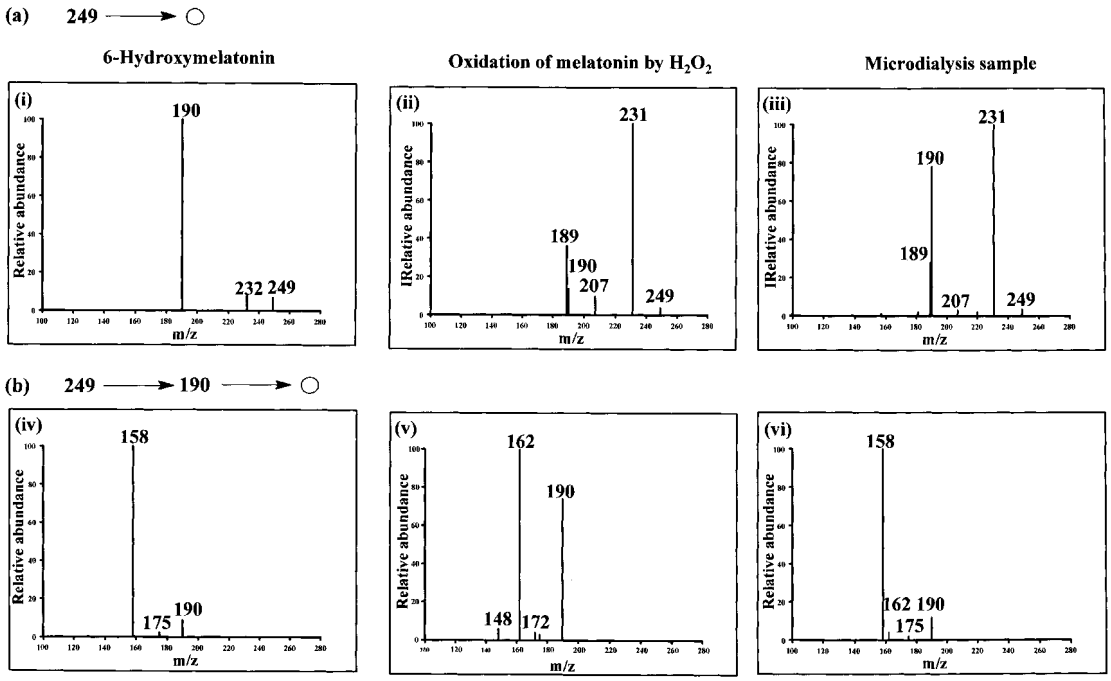

Figure 5. (a) MS/MS and (b) MS/MS/MS spectra of 6-hydroxymelatonin, oxidation product of melatonin by H_2O_2, and microdialysate sample at retention time of 3.6 min. The match of MS^3 spectra in (iv) and (vi) confirms that the product eluted at this time is 6-hydroxymelatonin.

tored (233 → products). Under these conditions, the mono- and di-oxidation products, and melatonin, have retention times of 5.1, 7.8 and 13.0 min (Fig. 4(a)), respectively. When the dialysate sample was analyzed under the same conditions, a chromatographic peak was also observed at 5.1 min. The MS/MS spectrum for this peak was identical to that of the mono-oxidation product (Figs 4(a) and (b)). This shows that the mono-oxidation product of melatonin was formed during melatonin metabolism. The structure of the mono-oxidation product of melatonin has been proposed as

the cyclic 2-hydroxymelatonin (structure II) using MS^4 experiments.[16] However, the di-oxidation product was not observed in the dialysate sample.

In Fig. 4(b) there is a broad peak (*m/z* 231) at 2.4–4.2 min. The MS/MS spectra at 3.6 min in Figs 4(a) and (b) are shown in Fig. 5 (ii and iii). The MS/MS spectrum of 6-hydroymelatonin (structure III), also with a retention time of 3.6 min, is shown in Fig. 5 (i). From these three MS/MS spectra, it seems that spectrum (iii) is the sum of spectra (i) and (ii). This means that the metabolite, 6-hydroxymelatonin, is co-eluted with another oxidation product of melatonin (an unknown broad peak). In order to confirm that the product eluted at 3.6 min is 6-hydroxymelatonin, MS^3 experiments were performed (Fig. 5 iv, v and vi). The MS^3 spectrum of 6-hydroxymelatonin matched that of the dialysate sample (iv and vi). The extra peak at *m/z* 162 in (vi) is due to the co-eluted oxidation product of melatonin (v). The two metabolites, cyclic 2-hydroxymelatonin and 6-hydroxymelatonin, identified in these experiments, have been found previously as metabolic products of melatonin.[17–19]

CONCLUSIONS

LC/MS/MS has been successfully coupled on-line to an *in vivo* microdialysis system for the monitoring of melatonin (unattended) for a period of 15 hours. Metabolites of melatonin were identified as 6-hydroxymelatonin and cyclic 2-hydroxymelatonin by off-line LC/MS^n experiments. These results show that *in vivo* microdialysis coupled on-line to LC/MS/MS is a promising method for studying pharmacokinetics in a freely moving animal, and that the MS^n capability of ion trap mass spectrometers is a versatile tool for structural analysis.

Acknowledgements

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