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Concurrent on-line sampling of melatonin in pineal microdialysates from conscious rat and its analysis by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

Dynamic changes of melatonin in microdialysates from the pineal gland of a freely moving rat were repeatedly determined by using on-line high-performance liquid chromatography with electrochemical detection. The detection limit for melatonin, *ca.* 5 pg, was well below that achieved with other systems. We observed a drastic increase of extracellular pineal melatonin during the transitional phase from the light period to the dark period. This application of microdialysis is a useful tool in the study of the physiological role of the mammalian pineal body.

INTRODUCTION

One of the neurochemical functions of the mammalian pineal organ is to transduce signals from the external environment via neural pathways to internal humoral factors [1,2]. One of the endocrinal outputs is melatonin, which plays a role as an internal zeitgeber [3]. Melatonin is synthesized from the amino acid tryptophan by a well-known biochemical process [4]. The indole metabolism of this process has a marked circadian change in response to external lighting conditions.

Various methods have been employed to determine pineal indoles [5–8]. Recently, melatonin has been determined by high-performance liquid chromatography (HPLC) with fluorimetric detection [9–11] or electrochemical detection (ED) [9,12–14]. Both methods exhibit good selectivity and high sensitivity for melatonin.

Brain microdialysis has been developed as a powerful technique for successive *in vivo* monitoring of extracellular chemical compounds in the micro-environment around nerve cells [15–17]. The electroactive compounds in the microdialysate are measurable by HPLC–ED, because this technique does not require clean-

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up of the dialysis perfusates before injection into the system. Also, brain microdialysis can be used to investigate the dynamic relationship between metabolic changes *in vivo* and ongoing behaviour, such as patterns of sleep and feeding, etc. [18,19].

In order to determine the dynamic changes of extracellular melatonin from the pineal gland of the freely moving rat under a 12-h light–dark cycle, we preliminarily attempted to use concurrent on-line sampling of pineal dialysate by the microdialysis method and its analysis by reversed-phase HPLC–ED. We also obtained, for the first time, a continuous series of chromatograms of the *in vivo* dialysates, which clearly indicate the appearance of a melatonin peak during the transitional phase from the end of the light period to the dark period.

EXPERIMENTAL

Reagents

Chemicals were obtained from the following sources: melatonin from Aldrich (Milwaukee, WI, U.S.A.); 5-methoxyindoleacetic acid (5MIAA) and 5-methoxytryptophol (5MTOH) from Sigma (St. Louis, MO, U.S.A.); 5-methoxytryptamin (5MT) from Fluka (Buchs, Switzerland); KH_2PO_4, H_3PO_4, CH_3COOH, HPLC-grade methanol and disodium ethylenediaminetetraacetic acid (EDTA) from Wako (Osaka, Japan); Ringer's solution (147 m*M* NaCl, 4 m*M* KCl, 4.5 m*M* $CaCl_2$, pH *ca.* 6.5) from Otsuka Pharmaceutical (Naruto, Japan). Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Chromatography

The HPLC apparatus (Eicom, Kyoto, Japan) was composed of a Model EP-10 pump (double-piston, reciprocating type), a Model DG-10 degasser, a Model PL-10 dampener, a Model AS-10 automatic injector, an SI-100 injector (both AS-10 and SI-100 contain Rheodyne 7125 injection valve), an Eicom-Prepak column (AC-ODS, 4 mm x 5 mm I.D.) and an Eicompak reversed-phase column (MA-ODS, 250 x 4.6 mm I.D., 7 μm particle size). During *in vivo* microdialysis, the microdialysate from the probe was collected every 19 min or 29 min in the 100-μl sample loop of the automatic injector, which was on-line with the HPLC system. Since analysis of melatonin was finished within 19 min, the sample loop was set to be held in the load position during 19 min or 29 min and was automatically switched to the injection position for 60 s, after which the cycle was repeated.

Electrochemical detection was accomplished with a Model ECD-100 amperometric detector (Eicom) equipped with a WE-3G graphite working electrode (Eicom) and an Ag/AgCl reference electrode.

The precolumn and the analytical column were housed in a water-circulated thermostat (Model CTC-100, Eicom), which enabled the temperature to be controlled between 10 and 40°C to ± 0.1°C. The separation of electroactive com-

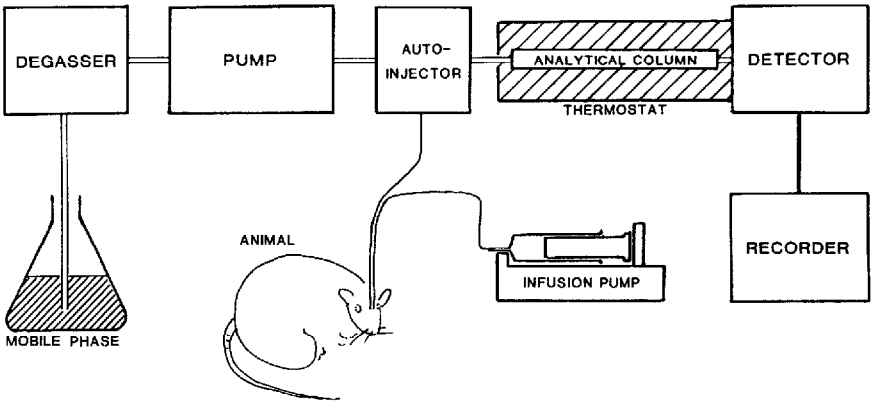

Fig. 1. Diagram of on-line microdialysis coupled to the HPLC–ED system.

pounds was carried out at 28°C. Detector signals for the separated compounds were recorded on a Chromatocorder-12 integrator (System-instruments, Tokyo, Japan). The complete system is illustrated in Fig. 1.

Mobile phase

A stock buffer (0.1 *M* KH_2PO_4, 0.05 *M* H_3PO_4, pH 3.1) was prepared as follows; 27.22 g of KH_2PO_4 and 1.96 g of H_3PO_4 were dissolved in 2.4 l of Milli-Q purified water. The mobile phase (34% v/v methanol, 4 μM EDTA) was prepared from 1.32 l of the stock buffer, 680 ml of HPLC-grade methanol and 0.8 ml of 10 m*M* EDTA solution. This mobile phase, deoxygenated by argon, was delivered at a flow-rate of 1.0 ml/min, producing a background pressure of *ca.* 98 bar in the pump.

Standard solutions

Stock solutions (10 mg/100 ml) of each compound were prepared in 0.01 *M* CH_3COOH and stored at −80°C. Prior to use, the stock solutions were diluted to appropriate concentrations with Ringer's solution.

Microdialysis technique

The dialysis probe was constructed in our laboratory according to the method of Nakahara *et al.* [20]. Two fused-silica tubes (0.075 mm I.D., 0.15 mm O.D., Eicom), one long (33 mm) and one short (23 mm), were inserted into a stainless-steel tube (10.5 mm x 0.55 mm O.D.). This assembly was fitted with a Cuprophan hollow dialysis fibre (3 mm x 0.2 mm I.D., 8 μm membrane thickness; Nikkiso, Tokyo, Japan), which was occluded at the tip with epoxy resin, leaving 1.5 mm available for diffusion. Free ends of the silica tubes, supported by metal tubing, formed the inlet and the outlet of the dialysis probe. The dialysis hollow fibre used in this experiment had a molecular mass cut-off of 5000.

The dialysis probe was continuously perfused at a rate of 1.5 μl/min with Ringer's solution via a PTFE tube (60 cm x 0.1 mm I.D., 0.4 mm O.D.) connected to a 5-ml Hamilton gas-tight syringe mounted on an infusion pump (Model 22, Harvard, U.S.A.). Samples were collected via the PTFE tube (60 cm long) in the 100-μl loop of the automatic injector as described under *Chromatography*. The travel time of the microdialysate from the dialysis probe to the end of the PTFE tube was *ca.* 6.5 min, measured by visually observing the appearance of the perfusate at the end of the PTFE tube.

The dialysis probe used in this experiment at room temperature had the following *in vitro* relative recoveries (mean ± S.D., $n = 4$):10.4 ± 2.2% for 5MT; 10.7 ± 2.4% for 5MTOH; 9.2 ± 2.3% for 5MIAA; and 9.9 ± 2.3% for melatonin.

Subjects and surgery

Male Wistar rats, 200–350 g, 8–13 weeks, were kept for at least 14 days under constant light(L)–dark(D) conditions (L:D 12:12, lights on at 07:00) with water and food *ad libitum* and at a controlled ambient temperature of 23 ± 2°C. Under pentobarbital anaesthesia (Nembutal 50 mg/kg i.p.), rats were mounted in the stereotaxic frame. According to Paxinos and Watson's atlas [21], a guide cannula (0.7 mm O.D.) for the microdialysis probe was implanted in the vicinity of the target point (A, 8.3 mm from bregma; L, 0 mm from mid-sagittal plane; V, 2.0 mm from skull surface) at a 45° angle to the frontal plane and at a 30° angle to the mid-sagittal plane.

After *ca.* 14 days of postoperative recovery each rat was placed in a microdialysis chamber, maintained at an ambient temperature of 23 ± 2°C and under a 12:12 L/D cycle (lights-on at 07:00, *ca.* 250 lx), for the *in vivo* microdialysis procedure. The microdialysis probe was inserted into the guide cannula, and then at least 3 h later, *in vivo* measurement was started as described above.

RESULTS AND DISCUSSION

Electrochemical characteristics

Hydrodynamic voltammograms (Fig. 2A and B) were generated for electrochemical characterizations of 5MT, 5MIAA, 5MTOH and melatonin. As shown in Fig. 2B, melatonin was oxidized in the potential range between +0.80 V and +0.95 V. In order to maximize the sensitivity for melatonin, the applied potential was maintained at +0.85 V *vs.* the Ag/AgCl reference electrode. Although all the potentials more than +0.90 V enhanced the sensitivity for melatonin, they also increased both the background current and the noise level. The high background current resulted in an unstable baseline, which was a disadvantage for constant monitoring *in vivo*.

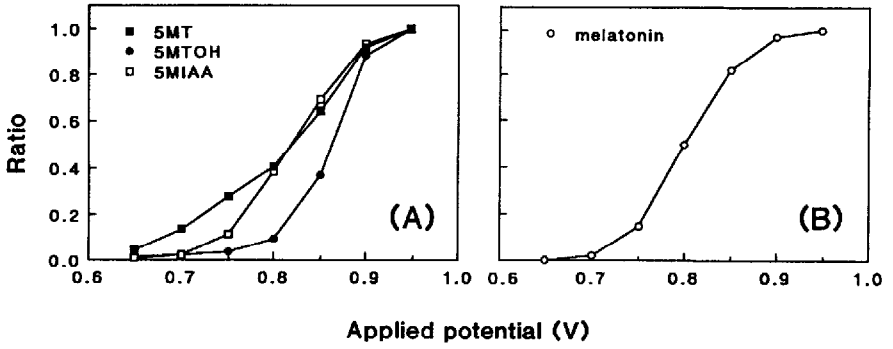

Fig. 2. Hydrodynamic voltammograms for 5MT, 5MTOH and 5MIAA (A), and melatonin (B). Abscissa: applied potential (V) *vs.* Ag/AgCl reference electrode; ordinate: ratio of response to maximal response at a given potential.

Detection limit

The detection limit, based on peak height *vs.* baseline noise ratio of 3:1, was *ca.* 3 pg for standard 5MT, 5MTOH and 5MIAA, and *ca.* 5 pg for standard melatonin. The detection limit obtained in this study was well below previously reported levels [9,10,12–14]. These results arose from the stable background current and the lower fluctuation of the solvent flow-rate, as well as the sensitivity of the working electrode.

Detector response and calibration

The detector response was tested using peak heights. It was linear in the range from 5 pg to 500 pg. The equations and correlation coefficients are given in Table I.

Stability of the HPLC–ED system

During *in vivo* experiments, it is necessary to monitor the states of the animal over 12 h to 24 h. In the course of the *in vitro* experiments, we evaluated the

TABLE I

EQUATIONS FOR THE CALIBRATION CURVE AND CORRELATION COEFFICIENTS FOR 5MT, 5MTOH, 5MIAA AND MELATONIN

The equation is $y = ax + b$, where x = amount of compound (pg) and y = peak current (nA); $n = 13$.

Compound	Range (pg)	Equation	
5MT	5–500	$y = 0.0038x + 0.034$	0.99994
5MTOH	5–500	$y = 0.0069x + 0.021$	0.99995
5MIAA	5–500	$y = 0.0061x + 0.006$	0.99996
Melatonin	5–500	$y = 0.0029x + 0.005$	0.99996

TABLE II

THE 24-h DETECTOR RESPONSE (MEAN ± S.D., $n = 48$) AND COEFFICIENTS OF VARIATION FOR THE COMPOUNDS IN STANDARD SOLUTION

Compound	Detector response (nA)	C.V. (%)
5MT	1.11 ± 0.05	4.18
5MTOH	1.54 ± 0.06	4.08
5MIAA	1.26 ± 0.07	5.35
Melatonin	1.22 ± 0.04	3.56

stability of the present HPLC–ED system, based on the reproducibility of the detector response for a standard solution. The standard solution of 6 pg/μl for 5MT, 5 pg/μl for 5MTOH and 5MIAA, and 10 pg/μl for melatonin was successively injected every 30 min over 24 h by using the automatic injector as well as *in vivo* experiments.

Table II shows the detector responses (mean ± S.D., $n = 48$) with the HPLC–ED system for the standard compounds, and their coefficients of variation (C.V.). The stability of the system in the *in vivo* chronic experiment is dependent on sampling by the microdialysis technique, as well as on the sensitivity of the working electrode [16]. The diffusion of the extracellular compounds across the dialysis membrane is prevented by tissue reactions around the membrane [16,22–24] and also by degeneration of the membrane itself [17,25,26]. In this study, the relative recovery ($n = 2$ probes) for melatonin was 12.0% just before the experiment and 9.3% immediately after 24 h of *in vivo* microdialysis. On the basis of the above results, the stability of the system appears to be more strongly related to the filtration of the dialysis membrane than to the sensitivity of the detector.

In vivo experiment

Fig. 3 shows chromatograms of the standard sample and a pineal dialysate. A peak corresponding to standard melatonin was seen at the retention time of 15.3 min in the chromatogram in Fig. 3B. The retention time of the peak with a different mobile phase, phosphate buffer–methanol (68:32, v/v), was 17.3 min as shown in Fig. 4A and B. Because of the correspondence in the chromatogram to the authentic melatonin with different mobile phases, the peak was identified as melatonin.

The amount (mean ± S.D.) of melatonin in a 43.5-μl microdialysate sample, perfused for 29 min, was 12.5 ± 0.6 pg during the light period, and 72.6 ± 48.8 pg, ranging from 30.7 ± 10.9 pg to 135.6 ± 92.1 pg, during the dark period. However, during the light period melatonin was not always detected in all rats. Some of the rats, whose melatonin level during the light period was below the detection limit, also showed a lower level of melatonin during the dark period. The melatonin

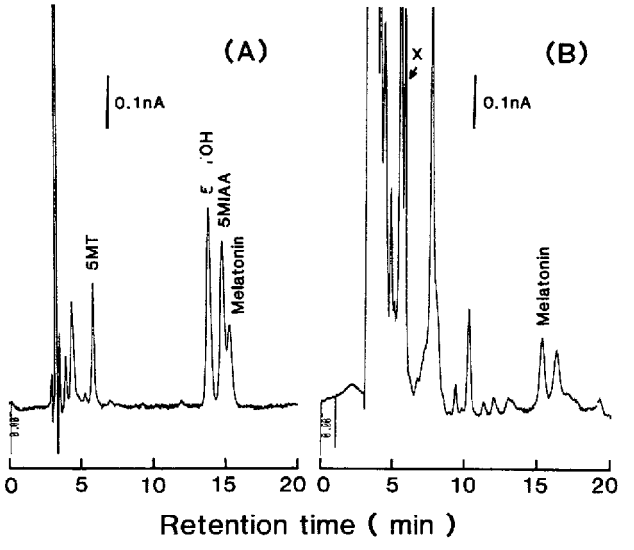

Fig. 3. Chromatograms of (A) the standard samples (each for 50 pg) and (B) a pineal dialysate sample obtained at midnight (dark period) from a freely moving rat. Retention times (min): (A), 5MT, 5.8; 5MTOH, 13.8; 5MIAA, 14.7; melatonin, 15.3; (B), 5MT, not identified; 5MTOH and 5MIAA, not detected; peak "X", an unknown peak that interfered with the identification of 5MT. Mobile phase, phosphate buffer (0.1 *M* KH_2PO_4, 0.05 *M* H_3PO_4)–34% (v/v) methanol containing 4 μM EDTA (pH 3.1); flow-rate, 1.0 ml/min; applied potential *vs.* Ag/AgCl electrode, +0.85 V.

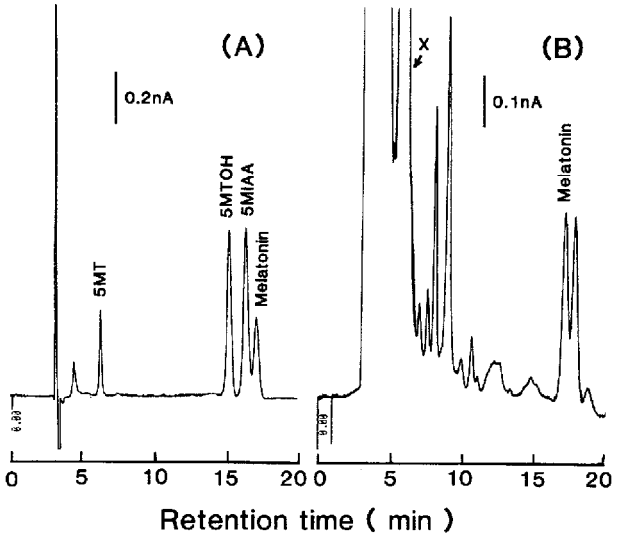

Fig. 4. Chromatograms of (A) the standard samples (each 100 pg) and (B) a pineal dialysate obtained at midnight from another freely moving rat. Retention times (min): (A), 5MT, 6.4; 5MTOH, 15.3; 5MIAA, 16.5; melatonin, 17.3; (B), similar to the results in Fig. 3B. Mobile phase, phosphate buffer–methanol (68:32, v/v). Other chromatographic conditions as in Fig. 3.

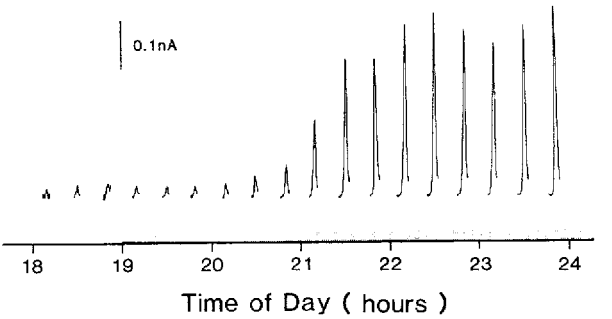

Fig. 5. Patterns of the pineal melatonin, sampled at 20-min intervals, over the transitional phase from the end of the light period to the dark period. These peaks were traced from a series of representative chromatograms from a freely moving rat. The dark period is indicated by a shaded bar just above the abscissa.

content in the dialysates varied in rats, since not all the dialysis probes were placed at the same site of the pineal gland.

Although extracellular melatonin was detected in the chromatogram of the dialysate at midnight (dark period), extracellular 5MTOH and 5MIAA were not simultaneously detected during both the dark and light periods. There may be several reasons for this: first, the concentrations of 5MTOH and 5MIAA in the pineal gland were only 2–5% of the concentration of melatonin reported by Mefford *et al.* [13]; and second, microdialysis has to be carried out on small samples containing compounds of interest in lower concentrations than the endogenous levels. Also, extracellular 5MT was not identified because it was modified by the presence of unknown compounds, as shown in Figs. 3B and 4B.

Fig. 5 illustrates a continuous series of melatonin peaks traced from representative chromatograms of the pineal dialysates. These peaks indicate a gradual appearance of the melatonin peak and its drastic increase over the transitional phase from the end of the light period to the dark period.

In summary, we reported here the first application of brain microdialysis coupled to a HPLC–ED system for the determination of *in vivo* extracellular melatonin in the rat pineal. This application not only offers the possibility of studying pineal neurochemical correlation with ongoing behaviour in the freely moving animal, but it can also contribute to the understanding of the transducing function of the pineal in mammalians.

NOTE

This animal research was designed and conducted on "Guiding Principles for Care and Use of Animals in the Field of Physiological Sciences, The Physiological Society of Japan, 1988".

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