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Long-term Automated HPLC Analysis of Microdialysis Samples from Multiple Freely Moving Animals

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Circadian rhythms are present in almost all organisms and biological processes. These daily rhythms control homeostasis and influence various aspects of life, including health, behavior and cognitive functions. The best marker for studying circadian rhythms is the hormone melatonin produced in the pineal gland of mammals. Unlike activity, heart rate or body temperature, melatonin production rhythm is very precise and is less influenced by hunger, sickness or stress.

To fully take advantage of melatonin as a circadian marker, the Borjigin Laboratory developed a method to automatically analyze consecutive samples of pineal dialysates from multiple rats. With the current system configuration, each rat is sampled every 20 minutes in experiments that can last up to two months.

The ability to frequently measure an individual's pineal secretion allows for the precise definition of melatonin onset and offset, the identification of individual differences, as well as detection of change in circadian phase under various experimental conditions. The automation and high throughput of this technique bypass the labor-intensive and error-prone manual handling of dialysates, and provide an accurate profile of daily melatonin production rhythm.

METHOD

Overview

The HPLC system consists of one Shimadzu SCL-10A VP controller, two Shimadzu LC-20AD isocratic pumps, a CTO-20AC column oven containing 2 Supelco C18 reversed phase columns, two RF-10AXL detectors, two VICI Cheminert® sample injectors (2-position/10-port actuator), and a VICI digital sequence programmer. Each system is designed to analyze pineal dialysates from four rats, with two rats to each detector.

As shown in Figure 1, detectors A and B analyze dialysates from rats A1 and B1 simultaneously for 10 minutes, then switch to rats A2 and B2. Each rat is thus analyzed every 20 minutes, and consecutive samples can be measured for up to two months.

Figure 1: VICI digital sequence programmer controls the 2-position/10-port actuator. Detector A analyzes dialysate from rat A1 while detector B simultaneously analyzes rat B1. Dialysates from rats A2 and B2 are then subsequently analyzed. The analysis switches back and forth between rat 1 and rat 2 every 10 minutes.
Sample Acquisition

Rats are implanted with microdialysis probes through their pineal gland. For a detailed description of probe construction and surgery, see (Borjigin and Liu 2008). Artificial cerebral spinal fluid is delivered to each implant via Instech peristaltic pumps, with two rats per pump at 2μl per minute. Each rat is linked to the peristaltic pump through a series of PEEK tubing connected through an Instech dual-channel swivel. The swivel is mounted on a counterbalance arm providing both vertical and horizontal mobility.

Freely moving rats tethered to the swivel are housed individually in cages situated in light controlled chambers. Dialysates are collected and delivered to the HPLC system through the sample injector (Figure 2). Two rats are connected to each sample injector. Staggered sample collection and analysis doubles the output of this system; while the dialysate from one rat is analyzed, the dialysate of the other rat is collected in the 20μL loop of the sample injector, with the excess running off into the waste. Every 10 minutes, the sequence programmer gives a signal to the fast microelectric actuator, and the previously collected dialysate is injected from the 2-position/10-port valve.

Data Analysis

For the purpose of circadian rhythm studies, the peaks of interest on a chromatograph are melatonin (MT) and its precursors, serotonin (5-HT) and N-acetylserotonin (NAS). These indoles are naturally fluorescent (Chin 1990). Separation of each sample is conducted by reversed phase C18 column, maintained at 45°C. The mobile phase is pumped at 1.5mL per minute, and consists of 34% methanol with about 10 mM sodium acetate. Due to slight differences in each system, the exact concentration of sodium acetate must be adjusted for each detector so that the NAS, 5-HT and MT peaks are present and distinct during each run.

The final adjusted retention times of the three peaks from nighttime pineal dialysates are shown in Figure 3. Note that dialysates are directly analyzed without any purification process, and it is of importance to ensure additional peaks do not interfere with the peaks of interest.

Data collection and sequence processing is performed on CLASS-VP firmware from Shimadzu. The sequence consists of two alternating methods set up for two detectors. Method 1 pertains to rat A1 and B1 and method 2 pertains to rat A2 and B2. The acquisition time window for each run is 8 minutes followed by 2 minutes of system equilibration, for a combined time of 10 minutes for each method.

The sequence is processed daily, and the resulting report is pasted into a preformatted Microsoft Excel worksheet. The baselines of certain MT peaks are checked and adjusted manually if necessary – these include the daily maximum value, as well as peaks during the rising and falling phases of MT production.
Conclusions

As other circadian markers such as activity, heart rate and body temperature could only give a rough estimate of daily rhythmic onset, the ability to distinctly and clearly identify both onset and offset of the melatonin rhythm for long periods of time within the same individuals offers many new insights into the circadian system.

Through this technique, individual circadian chronotypes can be identified (Liu and Borjigin 2006). Also, the duration in which an individual adjusts to a new schedule (commonly known as jetlag) can be clearly determined by noting the number of days it takes for both the MT onset and offset to re-stabilize after a change in the lighting schedule (Liu and Borjigin 2005). Individual differences in re-adjusting to jetlag can also be determined through this technique.

Furthermore, the dynamics in which the MT onset and offset independently shift during jetlag is interesting in and of itself, and may inform us of the molecular dynamics of the central circadian clock. Various types of drugs, such as isoproteronol, can be infused directly to the pineal gland through the microdialysis probes or delivered to animals via systemic injection, and their effects on the circadian system can be easily monitored across multiple cycles via the automated HPLC system.

Online microdialysis integrated with real-time HPLC analysis is a powerful tool for providing information on the circadian pacemaker, especially in combination with molecular and pharmacological tools. Other applications of this system are certainly possible for the long-term in-vivo analysis of various biological substances in freely moving animals.

Citations


