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Short communication

Simultaneous determination of serotonin, N-acetylserotonin and melatonin in the pineal gland of the juvenile golden hamster by high-performance liquid chromatography with electrochemical detection

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Abstract

A simple and simultaneous determination of melatonin and its precursors, serotonin (5-HT) and N-acetylserotonin, was achieved by reversed-phase high-performance liquid chromatography with electrochemical detection. The addition of an ion-paring agent, sodium 1-octanesulfonate, to the chromatographic mobile phase caused an increase of the retention time of 5-HT, and resulted in the successful simultaneous resolution of these three indoleamines. This method was used to quantitate these indoleamines in the pineal gland of juvenile golden hamsters.

Keywords: Serotonin; N-Acetylserotonin; Melatonin

1. Introduction

It is well established that melatonin is synthesized from serotonin (5-hydroxytryptamine; 5-HT) by a two-step process in the pineal gland [1]. The first step is N-acetylation of 5-HT by N-acetyltransferase to produce N-acetylserotonin (NAS). The second step is O-methylation of NAS to form melatonin by hydroxyindole-O-methyltransferase. Several methods, including radioimmunoassay and gas chromatography–mass spectrometry, have been developed to measure these indoleamines [2,3]. High-performance liquid chromatography (HPLC) with electrochemical detection has also been widely used to detect these indoleamines in physiological samples [2–12]. This method can detect multiple indoleamines on one chromatograph. However, different mobile phases have been required to resolve hydroxy- and methoxyindoleamines [4–6]. Since many hydroxyindoles, such as 5-HT and NAS, are more polar than methoxyindoles including melatonin, hydroxyindoles are generally eluted faster than methoxyindoles during reversed-phase HPLC. Therefore, the mobile phase used for the analysis of 5-HT and NAS

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contained lower concentrations of organic solvents, such as methanol and acetonitrile, than that used for melatonin analysis. It has been reported that ion-pairing agents affect the retention time of amines in reversed-phase HPLC [13–15]. Raynaud and Pévet [11] reported the simultaneous separation of four kinds of 5-methoxyindoleamines in the pineal gland by using these agents.

We report here a simple method for the simultaneous determination of 5-HT, NAS and melatonin using the ion-pairing agent, sodium 1-octanesulfonate (SOS), in reversed-phase HPLC with electrochemical detection. This method was used to measure both the hydroxy- and methoxyindoleamines in the pineal gland of juvenile golden hamsters (*Mesocricetus auratus*), one of the most studied animals in pineal research.

2. Experimental

2.1. Chemicals

Melatonin, 5-HT, NAS, 5-methoxyindole-3-acetic acid (MIAA), 5-methoxytryptophan (MTrp), 5-methoxytryptophol (MTPL) were obtained from Sigma (St. Louis, MO, USA). Tryptophan (Trp), hydroxytryptophan (HTrp), methanol, ethylenediaminetetraacetate tetrasodium salt (EDTA) and SOS were purchased from Nakarai (Kyoto, Japan), and 5-hydroxyindole-3-acetic acid (HIAA) from Tokyo Kasei (Tokyo, Japan). All other chemicals used were reagent grade. Water was purified with a Milli-Q system.

2.2. Apparatus

HPLC was carried out with a Model EP-10 liquid chromatography pump (EICOM, Kyoto, Japan) equipped with an Eicompack CA-5ODS reversed-phase column (150 × 4.6 mm I.D., 5-μm particles) and a guard column obtained from EICOM. The columns were kept at 25°C in a water bath. The electrochemical detector was an EICOM ECD-100 with a graphite carbon working electrode (EICOM WE-3G; 12 mm in diameter) and a Ag/AgCl reference electrode. Both a Hitachi D-200 integrator and a pen recorder were connected to the detector for recording.

2.3. Chromatography

The mobile phase contained 0.1 *M* sodium phosphate, 0.1 m*M* EDTA, 25% methanol (v/v) and the indicated concentration of SOS, pH 5.0 made fresh, filtered through a Millipore Millicup (0.45 μm), and degassed prior to each analysis. All separations were performed at a flow-rate of 1.0 ml/min.

2.4. Sample preparations

The golden hamsters used were bred in our colony. The animals were kept under a constant photoperiod (LD 14:10) at 23°C from birth. Light was on at 0600 and off at 2000. Juvenile golden hamsters (50–55 days of age) were sacrificed by decapitation at daytime (1300) and night time (0100 and 0430). The body weights of the hamsters were 97.48 ± 1.65 g. Pineal glands were removed quickly and homogenized in 100 μl of ice-cold 0.1 *M* perchloric acid. The homogenate was centrifuged at 10 000 *g* for 20 min at 4°C. The resultant supernatant solution (90 μl) was mixed with 10 μl of 1 *M* sodium phosphate, pH 4.3, and filtered through Millipore Samprep LCR4(T)-LG filters (0.22 μm). A 20-μl aliquot of the filtrate was injected into the chromatographic system with a Rheodyne injection valve (Model 7125). Since the concentration of 5-HT in the pineal gland was much higher than that of NAS and melatonin, quantitative determinations of 5-HT were made by comparing the peak area of the samples obtained from a Hitachi D-200 data integrator with those given by known concentrations of standards. For the quantitative determination of NAS and melatonin, the peak height of the samples obtained from the pen recorder was compared with those given by known concentrations of standards.

3. Results and discussion

Since the graphite carbon working electrode of the electrochemical detector limits methanol concentration in the mobile phase to less than 30% (v/v), we used 25% (v/v) methanol in the mobile phase for these experiments.

Fig. 1A shows the hydrodynamic voltammograms of 5-HT, NAS and melatonin. An applied potential greater than 900 mV resulted in higher sensitivity; however, the baseline noise also increased (Fig. 1B). Thus, the applied potential was maintained at 900 mV. At this potential, the detection limit of the indole amines was 5 pg (data not shown).

Fig. 2 shows the capacity factor of several indole amines in the reversed-phase column with different SOS concentrations in the mobile phase. The capacity factor of 5-HT was 0.6 in the absence of SOS, and increased to 8.5 when the concentration of SOS was raised to 5 m*M*. At this SOS concentration, however, the peak of 5-HT interfered with the peak of MTrp. Increasing the SOS concentration from 0 to 3 m*M* resulted in a slight decrease of the capacity factor of melatonin. The capacity factor of NAS, compared with that of 5-HT and melatonin, was unaffected by SOS at the concentrations we tested (0–5 m*M*). From these results, we chose 4 m*M* SOS in the mobile phase for HPLC analysis. At this SOS concentration, none of the indoleamines tested (Trp, HTrp, HIAA, MTrp, MIAA, MTPL) interfered with 5-HT, NAS or melatonin measurement. Although Goldman et al. [16] first reported a simultaneous detection of 5-HT and melatonin by cation-exchange HPLC, NAS was not resolved from the other indoleamines with their method. Fig. 3 shows the chromatograms of indoleamine standards (A) and a pineal gland extract of a juvenile golden hamster at 0430 (B).

Fig. 4 shows the amounts of 5-HT, NAS, and melatonin in the pineal glands of the juvenile

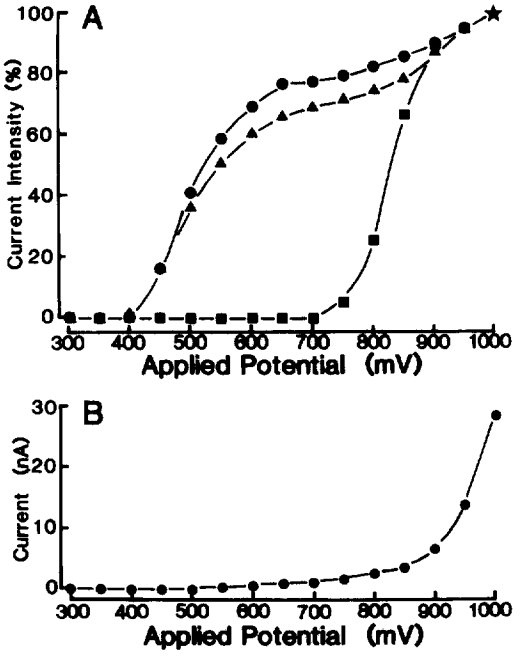

Fig. 1. (A) Hydrodynamic voltammogram for 5-HT (●), NAS (▲) and melatonin (■). Each point shows the percentage of the current relative to that at 1000 mV of applied potential (*). (B) Background current at each applied potential. The mobile phase was composed of 0.1 *M* sodium phosphate, 0.1 m*M* EDTA, 4 m*M* SOS and 25% methanol (v/v), pH 5.0.

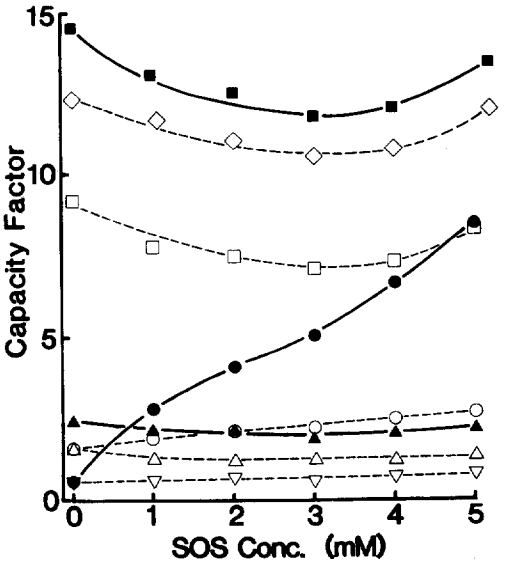

Fig. 2. Effect of SOS concentrations on the capacity factor of 5-HT (●), NAS (▲), melatonin (■), Trp (○), HIAA (△), MTrp (□), MIAA (▽) and MTPL (◇). Flow-through time was 1.59 min. The composition of the mobile phase (except for SOS) was the same as described in Fig. 1, with SOS added as indicated.

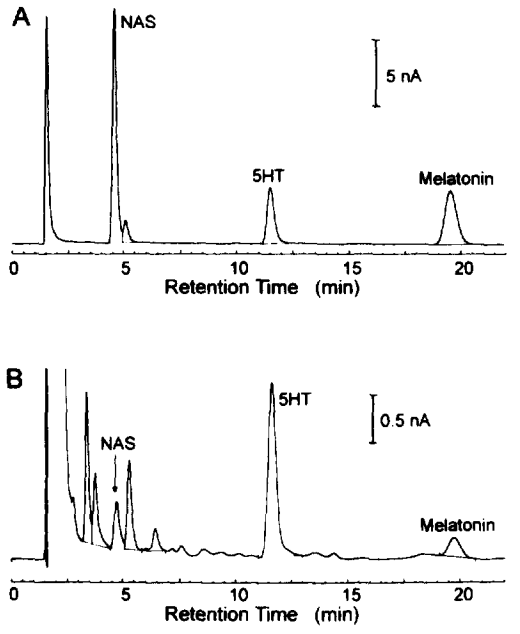

Fig. 3. (A) Chromatogram of indoleamine standards; 1 ng of each standard was injected. (B) Chromatogram of a pineal gland extracted from a juvenile golden hamster at night (0430). Mobile phase is the same as described in Fig. 1.

golden hamsters for both day and night. A significant decrease of 5-HT, and an increase of melatonin were observed at night. It has been suggested that the decrease of 5-HT in dark periods is primarily a consequence of the increase of melatonin production [12]. Melatonin levels in the pineal gland were 26.25 ± 3.11 pg at 1300, and increased to 52.78 ± 9.13 pg at 0100 and 245.78 ± 47.29 pg at 0430. An increase of the melatonin levels during the dark period reaching a maximum late in the dark period was also observed in adult golden hamsters [8,17]. Our results show that juvenile golden hamsters possess pineal melatonin rhythms similar to those of adult animals. However, pineal NAS levels, which are increased at night in the adult, did not show a significant rhythm in the juvenile.

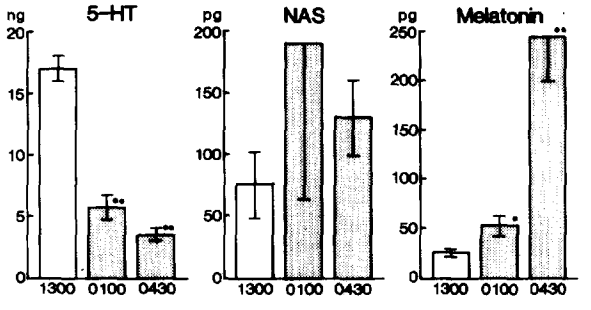

Fig. 4. Content of 5-HT, NAS and melatonin in the pineal gland of juvenile golden hamsters. Values are the average of six animals (1300 and 0100) or five animals (0430). Vertical bars indicate standard error. $^{*}P < 0.05$; $^{**}P < 0.001$.

Melatonin rhythms in the pineal gland are present in many mammals. However, some rodents and the pig are deficient in melatonin, or lack the melatonin rhythm in their pineal gland [18–20]. These results indicate that each species has its own regulatory system for pineal melatonin production. To understand the diversity and species specificity of the melatonin production system in mammals, analysis of melatonin and its precursors in the pineal gland should be examined further in other species. A simple and quick analytical method would facilitate the analysis of these indoleamines in a large number of samples. However, no effective simultaneous analysis of melatonin and its precursors, 5-HT and NAS, has been achieved. Here, we have described a fast simple HPLC method which enables the simultaneous measurement of these three indoleamines. This method is significantly important in the analysis of indoleamines of the pineal gland.

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