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Journal of Chromatography B, 692 (1997) 217–221

JOURNAL OF CHROMATOGRAPHY B

Short communication

Hydroxyindole-*O*-methyltransferase activity assay using high-performance liquid chromatography with fluorometric detection: determination of melatonin enzymatically formed from *N*-acetylserotonin and *S*-adenosyl-L-methionine

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Received 30 August 1996; revised 17 October 1996; accepted 4 November 1996

Abstract

A reliable, sensitive and rapid assay has been developed for determining the activity of hydroxyindole-*O*-methyltransferase (HIOMT; *S*-adenosyl-L-methionine:*N*-acetylserotonin-*O*-methyltransferase; EC 2.1.1.4), which catalyzes the final step in the melatonin (*N*-acetyl-5-methoxytryptamine) biosynthetic pathway. This method is based on the separation and detection of melatonin formed enzymatically from *N*-acetylserotonin and *S*-adenosyl-L-methionine, by high-performance liquid chromatography with fluorometric detection. The detection limit for melatonin formed per sample was as low as 150 fmol, indicating that the sensitivity of this assay was comparable to that of a radioisotopic assay. The assay was applied to the determination of HIOMT activity in rat pineal gland. The HIOMT activity obtained in this study was comparable with, or slightly lower than those reported previously using radioisotopic assays.

Keywords: Enzymes; Hydroxyindole-*O*-methyltransferase; Melatonin; *N*-Acetylserotonin; *S*-Adenosyl-L-methionine

1. Introduction

Hydroxyindole-*O*-methyltransferase (HIOMT; *S*-adenosyl-L-methionine: *N*-acetylserotonin-*O*- methyltransferase; EC 2.1.1.4) catalyzes the conversion of *N*-acetylserotonin to melatonin (*N*-acetyl-5-methoxytryptamine), a putative time-keeping hormone or neuromodulator in vertebrates [1,2]. HIOMT activity has been detected in the pineal gland, retina and Harderian gland of vertebrates [3–5].

Until now, HIOMT activity has been measured mainly by a radioisotopic assay that is based on *O*-methylation of *N*-acetylserotonin with *S*-[^{14}C]- or *S*-[^{3}H]methyl]adenosyl-L-methionine as the methyl donor and organic extraction of enzymatically-formed radioactive melatonin [6,7]. The radioisotopic assay is sensitive, but requires expensive radioactive substrate and complicated postincubation sample preparation by organic extraction. More simple and reliable techniques such as high-performance liquid chromatography (HPLC) with fluorometric detection have recently become available for the determination of melatonin and its

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PII S0378-4347(96)00503-8

related indole compounds [8–11]. In the present study, we developed a non-radioisotopic assay of HIOMT activity using rat pineal gland as enzyme source. The HIOMT activity assay is based on separation and detection of melatonin formed enzymatically from *N*-acetylserotonin and *S*-adenosyl-L-methionine, using HPLC-fluorometric detection. The method enables a simple and accurate determination of HIOMT activity.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a delivery pump KHP-010 (Kyowa Seimitu, Tokyo, Japan), a Rheodyne injection valve Model 7725i (Rheodyne, Berkeley, CA, USA) with a 20 or 50 μl sample loop, a Superiox ODS S-5 μm column (150×4.6 mm I.D., Shiseido, Tokyo, Japan), and a fluorometric detector, RF-550 (Shimadzu, Kyoto, Japan). The detector was used with excitation and emission wavelengths set at 280 and 340 nm, respectively. Chromatograms were recorded and processed using a Chromatopack C-R5A (Shimadzu).

2.2. Chemicals

All of the reagents used were of the highest purity available, and were obtained from commercial sources. Melatonin, its structurally related indole compounds, and *S*-adenosyl-L-methionine were purchased from Sigma (St. Louis, MO, USA).

2.3. Animals

Six-week-old female Wistar–Imamichi-derived rats were used. Rats were entrained to a 12 h light:12 h dark lighting schedule and killed by decapitation during the light period. Pineal glands were removed rapidly, frozen on solid CO_2 and stored at −80°C until they were assayed.

2.4. HIOMT activity assay

Rat pineal glands were homogenized in various volumes, depending upon the sample and protein concentration, of ice-cold 0.05 *M* sodium phosphate buffer (pH 7.9). Homogenates were centrifuged at 12 000 *g* for 10 min at 4°C. Volumes (55 μl) of the supernatant were mixed with 25 μl of 3.2 m*M* *N*-acetylserotonin and 0.32 m*M* *S*-adenosyl-L-methionine in 0.05 *M* sodium phosphate buffer (pH 7.9) and incubated at 37°C. The final concentrations of these substrates were 1 m*M* and 0.1 m*M*, respectively.

The enzymatic reaction was stopped by the addition of 20 μl of 6 *M* perchloric acid. After centrifugation at 12 000 *g* for 10 min at 4°C, the supernatant was filtered through a 0.2 μm sumplep LG4 filter (Millipore). The filtrate (2–10 μl) was subjected to the HPLC analysis. As controls, reaction mixtures were incubated either without *N*-acetylserotonin or without enzyme, and were analyzed using HPLC.

The HPLC mobile phase consisted of 0.05 *M* ammonium acetate and 30% methanol (v/v), adjusted to pH 4.3 using acetic acid, and was pumped at a flow-rate of 0.7 ml/min and 25°C. Peaks were identified according to retention time and melatonin was quantified according to peak height; the calibration curve was calculated by analyzing known amounts (150 fmol to 2.5 pmol) of melatonin.

To determine recovery of melatonin through extraction procedure and HPLC analysis, 1.5 and 2.5 pmol of melatonin were added to pooled rat pineal homogenates. After centrifugation, the supernatant (55 μl) was mixed with 25 μl of the substrate solution of HIOMT assay and 20 μl of 6 *M* perchloric acid. The mixtures were centrifuged and 50 μl of the supernatant were analyzed by HPLC.

Intra- and interassay variation of coefficients in HPLC analysis were determined using quality control samples composed of pooled rat pineal homogenates or 0.5 and 2.0 pmol of melatonin.

2.5. Protein assay

Protein content was determined using a dye-binding method [12], and using bovine serum albumin as a standard.

3. Results

On the chromatogram of a standard solution, the melatonin peak was separated clearly from those of eight other indole compounds under the HPLC

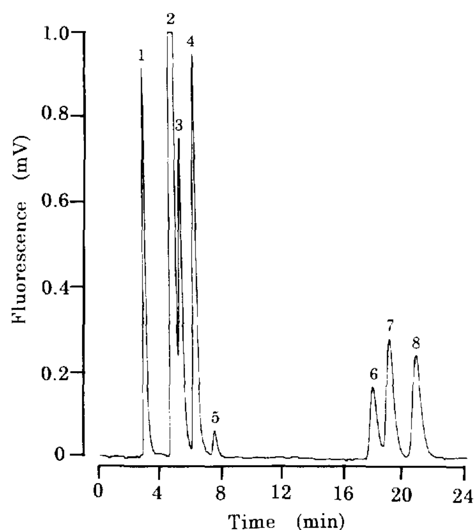

Fig. 1. Chromatogram of a standard solution containing 250 pg (1.0–1.3 pmol) of the following compounds. Peaks: 1=5-hydroxytryptamine (serotonin), 2=5-hydroxyindole-3-acetic acid and 5-hydroxytryptophol, 3=N-acetylserotonin, 4=5-methoxytryptamine, 5=6-hydroxymelatonin, 6=5-methoxyindole-3-acetic acid, 7=5-methoxytryptophol, 8=N-acetyl-5-methoxytryptamine (melatonin).

conditions used (Fig. 1). The calibration curve was linear in the range of 150 fmol to 2.5 pmol of melatonin. The correlation coefficient was 0.997, and the detection limit was as low as 150 fmol for a 2:1 signal-to-noise ratio. Intra- and interassay variation coefficients in HPLC analysis were 4.6% ($n=5$) and 6.3% ($n=5$), respectively.

The typical HPLC elution profile obtained from reaction mixture of rat pineal gland homogenate with N-acetylserotonin and S-adenosyl-L-methionine is shown in Fig. 2A. The large amount of the substrate N-acetylserotonin did not interfere with the detection of melatonin. A small peak of melatonin was detected on the chromatogram obtained from a reaction mixture of rat pineal gland homogenate without N-acetylserotonin (Fig. 2B). Peaks with identical retention times to those of other indole compounds were also observed on both chromatograms. In mixtures of substrates without the enzyme source, no melatonin peak was detected using HPLC analysis (data not shown).

The recovery (mean±SEM of $n=4$) of melatonin through the extraction procedure and HPLC analysis

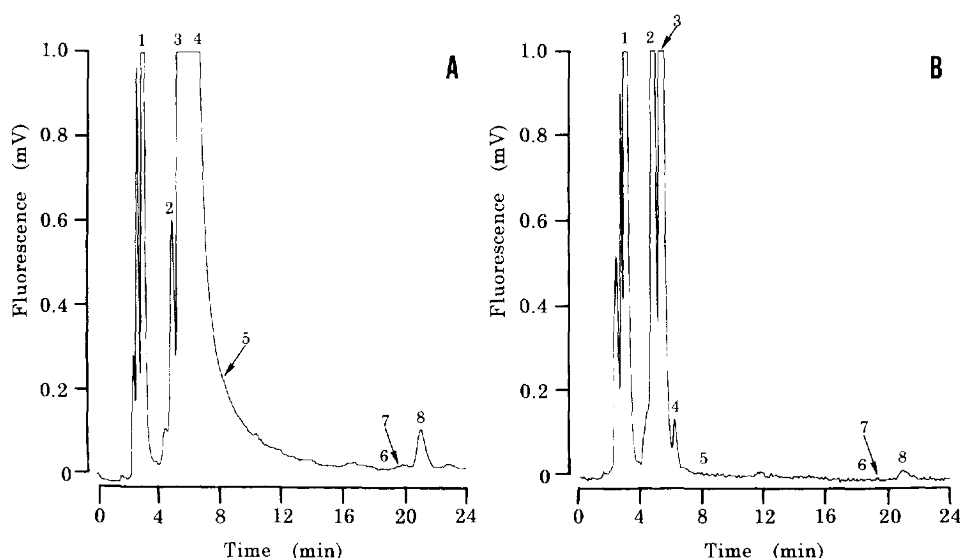

Fig. 2. Chromatograms obtained from reaction mixtures in which rat pineal gland was the enzyme source: (A) experimental incubation with N-acetylserotonin and S-adenosyl-L-methionine and (B) control incubation without N-acetylserotonin. The numbers in the figure indicate the elution position of standards (see Fig. 1). Single pineal gland was homogenized in 0.4 ml of ice-cold 0.05 M sodium phosphate buffer (pH 7.9). After centrifugation, 55 μl of the resulting supernatant was assayed for HIOMT activity (30 min incubation at 37°C).

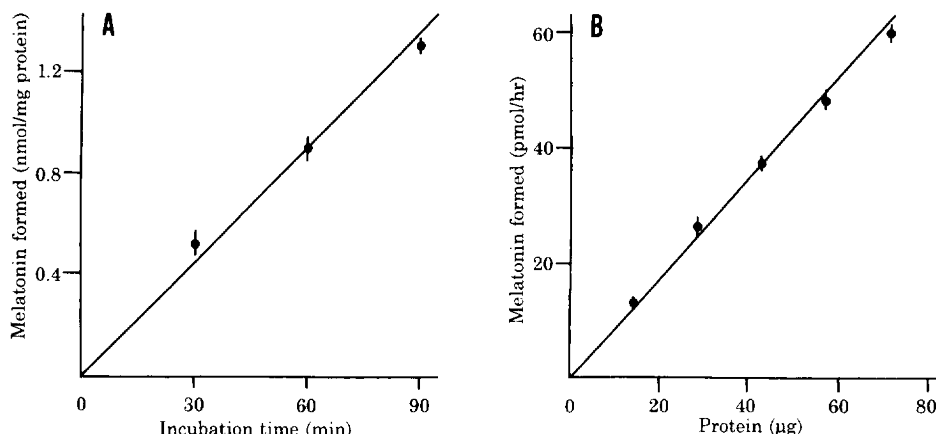

Fig. 3. (A) Time-course of *O*-methylation of *N*-acetylserotonin using rat pineal gland homogenate as the enzyme source. Five pineal glands were homogenized in 1.0 ml of ice-cold 0.05 *M* sodium phosphate buffer (pH 7.9). The enzyme reaction was carried out for various times at 37°C. The final concentrations of two substrates, *N*-acetylserotonin and *S*-adenosyl-L-methionine, were 1 m*M* and 0.1 m*M*, respectively. (B) Effects of protein concentration of rat pineal gland on the amount of enzymatically-formed melatonin. Seventeen pineal glands were homogenized in 1.0 ml of ice-cold 0.05 *M* sodium phosphate buffer (pH 7.9). After centrifugation, the resulting supernatant (protein concentration 1.3 μg/μl) was diluted at different ratios with the homogenization buffer, and assayed for HIOMT activity (1 h incubation at 37°C). The substrates were used at the same concentrations as those in (A). In both (A) and (B), each point and vertical line indicates the mean±SEM of triplicate determinations.

was 97.3±0.7%. No significant change in melatonin levels in the extract of reaction mixtures was observed for 24 h at −20°C, indicating that melatonin in the extract is stable for at least 24 h at −20°C. We kept the sample at −20°C before HPLC analysis and analyzed the sample by HPLC within 24 h after extraction.

The rate of melatonin formation proceeded linearly for 90 min at 37°C (Fig. 3A). HIOMT activity in rat pineal glands as function of enzyme concentration, is shown in Fig. 3B. A linear relationship was observed between the amounts of protein in the rat pineal glands (14.3–71.5 μg) and those of melatonin formed. HIOMT activities (mean±SEM of $n=5$) in rat pineal glands were 58.6±1.4 pmol/h per gland or 903.1±20.6 pmol/h per mg protein.

4. Discussion

HIOMT catalyzes the conversion of *N*-acetylserotonin to melatonin by *O*-methylation of the 5-hydroxy group, using *S*-adenosyl-L-methionine as the methyl donor [1,2]. In the study described here, reversed-phase HPLC coupled with fluorometric detection was employed to separate and detect melatonin formed enzymatically from *N*-acetylserotonin and *S*-adenosyl-L-methionine. This method allows the determination of HIOMT activity to be made with high specificity, sensitivity and accuracy. In addition, this HIOMT activity assay has the following advantages relative to the radioisotopic assays: (i) it is more economical than radioisotopic assays, due to the high cost of isotopically-labeled *S*-adenosyl-L-methionine and (ii) postincubation sample preparation is simpler; samples are deproteinated using perchloric acid and centrifugation, and the supernatants are directly applied to HPLC.

In the present study, HIOMT activity in rat pineal glands was comparable with, or slightly lower than those reported previously using radioisotopic assays [7,13,14]. The HPLC-fluorometric detection assay described here is specific, because it only measures enzymatically formed melatonin. As can be seen in the chromatograms in Fig. 2, methoxyindole compounds (e.g., 5-methoxytryptamine) other than melatonin appear to be formed. It has been reported that other hydroxyindoles present in the pineal gland including 5-hydroxyindoleacetic acid, 5-hydroxytryptaphol and 5-hydroxytryptamine (serotonin) are

methylated [4,7,15]. It is possible that the radioisotopic assays are not specific, although most of the other methoxylated indole compounds are removed by organic extraction [7,15,16].

The assay method described here is a suitable technique for determining HIOMT activity in different tissues, and will allow a greater understanding of the regulation of melatonin production.

Acknowledgments

This work was supported in part by a grant from the Japanese Ministry of Education, Science and Culture, to M.T.I.

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