Introduction

Melatonin, N-acetyl-5-methoxytriptamine, is a pineal hormone synthesized from L-tryptophan via serotonin and N-acetylserotonin. The synthesis and the secretion of melatonin, which is regulated by a light-dark signal and the enzymatic activity of serotonin-N-acetyltransferase, shows a clear circadian rhythm increasing during the night. Melatonin thus transmits a light-dark signal to various brain and peripheral tissues via blood circulation. In the suprachiasmatic nuclei, the melatonin receptor is also expressed, and melatonin is reported to regulate the biological clock in this tissue.

Recently, the lifestyle in human society has become complicated, and rhythm disorders, such as obstructive sleep apnoea syndrome and non-24-hour sleep-wake syndrome, are increasing. For the treating these rhythm disorders, determining of the melatonin rhythm in each patient is expected as an indicator of individual biological rhythms. Evaluating the melatonin rhythm in each patient is also clinically useful for chronotherapy, which enhances the efficacy of the medicine and reduces the side effects by considering the biological rhythm. However, the amount of endogenous melatonin is extremely low, and a 1 – 5 mL blood sample is needed for the determination of melatonin by the methods already reported. To obtain 1 – 5 mL of blood, blood sampling from the cubital vein is normally required, and it is practically a severe task for patients to measure the circadian rhythm (blood should be obtained 6 – 8 times within one day). The limit of detection of melatonin using this system is about 200 amol/injection, and the determination of endogenous melatonin in a small volume of human physiological fluids, such as 100 μL of plasma and 300 μL of saliva, was successfully accomplished.

Keywords Melatonin, column-switching, fluorescence, on-line oxidation, HPLC

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injection, therefore, a few mL of blood and saliva are needed. On the other hand, we have already reported a highly sensitive HPLC method for the determination of melatonin using precolumn oxidation and fluorescence detection.31–34 By this method, melatonin is converted to an oxidized compound, \( N\)-[(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl] acetamide (6-MOQMA, Fig. 1), and the produced 6-MOQMA has a strong fluorescence emission at 382 nm with excitation at 246 nm. The limit of detection of melatonin using this precolumn oxidation method was 200 amol/injection using a semimicro-HPLC system. However, this method requires skillful techniques to obtain reproducible results, and also to reduce contamination from the environment; therefore, the development of a fully-automated on-line oxidation HPLC system is highly required as a practically useful method. In the present study, we developed a fully-automated on-line oxidation column-switching HPLC system, and the amount of endogenous melatonin in human clinical samples (plasma and saliva) has been determined.

Experimental

Materials

Melatonin was purchased from SIGMA (St. Louis, MO). Methanol (MeOH) and tetrahydrofuran (THF) of HPLC grade, ethanol (EtOH, dry, 99.5%) trifluoroacetic acid (TFA), sodium carbonate (Na\(_2\)CO\(_3\)) and aqueous hydrogen peroxide (H\(_2\)O\(_2\), 31%, v/v) were obtained from Wako (Osaka, Japan). Acetonitrile (MeCN) of HPLC grade was obtained from Nacalai Tesque (Kyoto, Japan). Water was purified using Milli-Q Elix3 and Gradient A 10 systems (Merck Millipore, Billerica, MA). All other reagents were of guaranteed reagent grade and used without further purification.

Effect of organic solvents on the oxidation of melatonin

Melatonin was dissolved in water containing an organic solvent (MeOH, EtOH, MeCN or THF; concentration of the organic solvent was 1.2, 2.4, 6 or 12%). To a glass vial, 1.2 nM of the melatonin solution (100 \( \mu \)L), an aqueous 120 mM Na\(_2\)CO\(_3\) solution (10 \( \mu \)L) and aqueous 1.2 mM H\(_2\)O\(_2\) solution (10 \( \mu \)L) were added. The vial was tightly capped and heated at 100 \( ^\circ \)C for 30 min; then, 20 \( \mu \)L of the reaction mixture was injected into a reversed-phase HPLC system. The HPLC system (NANOSPACE SI-2 series, Shiseido, Tokyo, Japan) consisted of a type 3009 degasser, a 3101 pump, a 3023 auto sampler, a 3004 column oven and a 3213 fluorescence detector. The analytical column was a CAPCELL PAK C18 MGII (75 mm \( \times \) 2.0 mm i.d., Shiseido), and the mobile phase was a MeCN-TFA-water (10:0.08:90, v/v, 200 \( \mu \)L/min). Fluorescence detection was carried out at 400 nm with excitation at 245 nm.

Establishment of an on-line oxidation unit

The on-line oxidation unit consisted of two 3101 pumps, a 3011 high-pressure valve and a 3019 thermo reactor (NANOSPACE SI-2 series, Shiseido). The flow diagram of the HPLC system used for the evaluation of this on-line oxidation unit is shown in Fig. 2. The eluent of pump 1 was pure water. The reagent added from pump 2 was aqueous Na\(_2\)CO\(_3\), and aqueous H\(_2\)O\(_2\) was added from pump 3. The melatonin solution (10 nM, 50 \( \mu \)L) was injected from the auto sampler, and mixed with the aqueous Na\(_2\)CO\(_3\) and H\(_2\)O\(_2\) solutions. The mixture was introduced into the thermo reactor by switching high-pressure valve 1 and sealed by switching high-pressure valve 1 again,
then heated for 30 - 90 min. The reaction mixture was then introduced into the concentration column (Asahipak ODP 40-2T, 35 mm x 2.0 mm i.d., 25°C, Shodex, Tokyo, Japan) by switching the high-pressure valves 1 and 2, and washed with pure water (100 μL min⁻¹, 20 min) provided by pump 1. The concentrated 6-MOQMA was then introduced into the analytical column (CAPCELL PAK C18 ACR, 150 mm x 1.0 mm i.d., 40°C, Shiseido) by switching high-pressure valve 2. The mobile phase of the analytical column was MeCN-TFA-water (12:0.08:88, v/v, 50 μL min⁻¹), and the fluorescence detection of both 6-MOQMA and melatonin was carried out at 370 nm with excitation at 278 nm.

Sample preparation procedure of human physiological fluids

Human blood was collected in a heparinized tube (Nippon Becton Dickinson, Tokyo, Japan), and the plasma was obtained by centrifugation (12100g, 4°C) for 10 min. Saliva was directly collected in a clear tube. Aliquots of the plasma (100 μL) and saliva (300 μL) were applied to a solid-phase extraction cartridge (adandé:l PEP0301, Shiseido) and washed with 5 mL of water. The retained components were then eluted by adding 300 μL of MeCN, and the eluate was dried. The residue was dissolved in 60 μL of water, and 50 μL of the solution was injected into the fully-automated on-line oxidation column-switching HPLC system described in the following section.

A fully-automated on-line oxidation column-switching HPLC system

The fully-automated on-line oxidation column-switching HPLC system (NANOSPACE SI-2 series, Shiseido) consisted of a 3202 degasser, six pumps (3101 and 3001), a 3023 autosampler, two 3004 column ovens, two 3213 fluorescence detectors, three high-pressure valves (3011 and 3012) and a 3019 thermo reactor with a reaction loop of 500 μL. The flow diagram is shown in Fig. 3. The sample solution was injected from an auto sampler, and separated by a purification column (Proteonavi, 75 mm x 1.0 mm i.d., 60°C, Shiseido) using pure water as the mobile phase (100 μL min⁻¹). Fluorescence detection of the melatonin was carried out at 350 nm with excitation at 278 nm, and the melatonin fraction was collected in a loop by switching high-pressure valve 1. The collected melatonin fraction was introduced into the thermo reactor by switching high-pressure valve 1 again (eluent of pump 3 was pure water, 100 μL min⁻¹), and the derivatizing reagents (aqueous 25 mM Na₂CO₃ with the flow rate of 80 μL min⁻¹ and aqueous 2 mM H₂O₂ with the flow rate of 20 μL min⁻¹) were added from pumps 4 and 5. The thermo reactor was then tightly sealed by switching high-pressure valve 2, and the mixture was heated at 110°C for 30 min. The produced 6-MOQMA was trapped on a concentration column (Asahipak ODP 40-1T, 35 mm x 1.0 mm i.d., 25°C, Shodex) by switching high-pressure valves 2 and 3 (the mobile phase was pure water from pump 3, 100 μL min⁻¹). The concentrated 6-MOQMA was then introduced into the analytical column (CAPCELL PAK C18 IF, 250 mm x 1.0 mm i.d., 50°C, Shiseido) by switching the high-pressure valve 3 with the mobile phase of MeCN-TFA-water (16:0.08:84, v/v, 20 μL min⁻¹) and determined by a fluorescence detector. Fluorescence detection of 6-MOQMA was carried out.
at 400 nm with excitation at 245 nm.

**Results and Discussion**

**Effect of organic solvents on the oxidation of melatonin and selection of the purification column**

For the determination of endogenous melatonin in a small clinical sample volume, a highly sensitive and selective analytical method is essential. For selective analysis, the on-line purification of melatonin in the physiological samples is effective, and the pre-column oxidation of melatonin to a highly-fluorescent compound, 6-MOQMA, is useful for sensitive determination. For the purification of melatonin in biological matrices using a reversed-phase column, some organic solvents are frequently used as the mobile phase additive; these organic solvents may affect the recovery of the on-line oxidation of melatonin. Therefore, purification columns and mobile phases used for the reversed-phase isolation of melatonin have been investigated. As the organic solvents, MeOH, EtOH, MeCN and THF were selected, and the effect of these solvents on the oxidation of melatonin was investigated. As a result, the oxidative reaction was severely affected by the addition of these organic solvents, and only sufficiently proceeded in aqueous solutions containing 0 – 2% MeCN (Fig. 4).

Therefore, mild reversed-phase columns, such as the CN, C1 and C4 columns, were examined as the purification column, because weak mobile phases (aqueous solutions containing 0 - 2% MeCN) could be used for compatibility with the on-line oxidation. A C4 column showed a good separation, and the sufficient resolution of melatonin could be obtained compared to the CN and C1 columns. Based on these findings, a C4 column of 1.0 mm i.d. x 75 mm was used to inhibit the diffusion and to decrease the volume of the melatonin fraction; a column length of 75 mm and a temperature of 60°C were used for shortening the analytical time.

For the determination of melatonin by reversed-phase HPLC, several methods have already been reported. In these methods, a high concentration of organic solvents, such as 20 – 50% of MeOH or MeCN, was required as the mobile phase. However, melatonin could not be oxidized in these mobile phases containing a high concentration of organic solvents.

Therefore, in the present investigation, the solvents for the pre-column oxidation were thoroughly examined. It was found that the oxidative reaction sufficiently proceeded in aqueous solutions containing 0 – 2% MeCN. By using aqueous 0 – 2% MeCN as the mobile phase and a weak reversed-phase C4 column as the stationary phase, the purification of melatonin in human clinical samples could be successfully accomplished.

**Design and establishment of an on-line oxidation unit**

The oxidative conversion of melatonin to 6-MOQMA is useful for a highly sensitive analysis of melatonin. To establish a fully-automated system applying this concept, an on-line oxidation unit for the pre-column oxidation of melatonin is necessary. In the present study, we designed and developed the unit illustrated in Fig. 2, and the on-line oxidation conditions were investigated. Melatonin was injected from the auto-sampler, mixed with oxidation reagents (H2O2 and Na2CO3) and introduced into the thermo reactor. The thermo reactor was tightly sealed by switching HPV1 and 2, and the on-line oxidation was carried out by heating the reactor. After the oxidation, 6-MOQMA formed by the reaction was trapped using the concentration column (Asahipak ODP, an alkaline resistant reversed-phase column) and the reagents were removed. The concentrated 6-MOQMA was then injected into the CAPCELL PAK C18 ACR column and determined by the fluorescence detector. The remaining melatonin could also be simultaneously determined by the same HPLC system. The reaction conditions were investigated by changing the concentrations of Na2CO3 and H2O2, the reaction temperature and the reaction time. The amount of 6-MOQMA increased along with the increasing concentrations of Na2CO3 and H2O2. However, the amount of 6-MOQMA decreased in the presence of excess Na2CO3 and H2O2. Concerning the reaction temperature, the oxidative reaction was effectively proceeded by increasing the temperature. However, 6-MOQMA was not stable in the reaction mixture with the temperatures above 120°C. As a result, 6-MOQMA was produced in good yield (around 80%) using 40 mM Na2CO3 and 0.8 mM H2O2 as the oxidation reagents, and heated at 110°C for 30 min. Figure 3 shows chromatograms of 6-MOQMA and melatonin with/without oxidation. Without oxidation, a clear peak of melatonin was observed at 25 min and 6-MOQMA was not present. On the other hand, the melatonin disappeared after oxidation, and a clear peak of 6-MOQMA was observed at 12 min.

In previous reports, we have already described the oxidation...
Development of a fully-automated on-line oxidation column-switching HPLC system

According to the results shown above, a fully-automated on-line oxidation column-switching HPLC system was established. The flow diagram is illustrated in Fig. 3. This system consists of four steps. In the first step, melatonin was fractionated using a C4 reversed-phase column (Proteonavi, 75 mm × 1.0 mm i.d.), with pure water as the mobile phase. In the second step, the obtained melatonin fraction was on-line collected and oxidized to 6-MOQMA by mixing the reagents and heating at 110 °C for 30 min. In the third step, the produced 6-MOQMA was concentrated by a short alkaline resistant C18 trapping column, and excess reagents were removed. 6-MOQMA was then introduced into the microbore-ODS column and sensitive determination was performed in the final fourth step. To develop a sensitive fully-automated HPLC system, a concentration column and an analytical column were investigated. As the concentration column, Asahipak ODP columns with different dimensions (1.0 mm × 35 mm, 1.0 × 50, 2.0 × 35, 2.0 × 50), were tested. As a result, the highly sensitive analysis of 6-MOQMA could be performed by an Asahipak ODP column of 1.0 mm i.d. × 35 mm length. Concerning the analytical column (1.0 mm i.d. × 250 mm), CAPCELL PAK C18 UG120, MG-II (particle size, 3 μm) and IF columns (1.8 μm) were tested. The CAPCELL PAK C18 IF column was selected as the analytical column with its high theoretical plate number. Combining these columns and solvents, a fully-automated on-line oxidation column-switching HPLC system was developed.

The present method was validated by checking the calibration line, intra-day precision and inter-day precision using the standard melatonin. The intra-day precision, inter-day precision and accuracy using the human plasma and saliva were also examined. The calibration line (γ = 1.29x + 0.64, where x is the amount of injected melatonin (fmol), γ is the fluorescence intensity (mV) of final detector) was linear from 500 amol to 50 fmol with a correlation coefficient greater than 0.9997. The intra-day precision and the inter-day precision of the standard melatonin were 0.86% (RSD, n = 5) and 4.07% (RSD, 5 days), respectively. The intra-day precision, inter-day precision and accuracy using endogenous melatonin in human plasma and saliva were also examined. As a result, the RSD values of the intra-day precision and the inter-day precision using human plasma were 9.95 and 8.63%, and those for human saliva were 2.44 and 4.83%, respectively. The accuracy of the melatonin measurement in human plasma and saliva using the present method was determined by comparing the slope of the calibration line and those of the standard lines constructed by adding 0.5, 1, 5, 10 and 50 fmol of melatonin to the human plasma or saliva. The accuracy values were 106 and 103%, respectively. The lower limit of quantification and the lower limit of detection of melatonin using the present method were 500 amol (injection amount) and 200 amol (S/N = 3, injection amount), respectively.

Until now, various analytical methods, such as enzyme immunoassay, GC-MS and HPLC methods, have been reported for the determination of melatonin. Enzymatic methods (both RIA and ELISA) are frequently used, and various assay kits are commercially available. However, enzymatic immunoassays are widely known to show cross-reactions or non-specific bindings, especially for the determination of trace amounts of an analyte like melatonin. GC-MS methods with N-trimethylsilyl or pentafluoropropionic derivatization are sensitive, however, both derivatization procedures require skillful techniques. Concerning HPLC, various methods combining an electrochemical detector, a fluorescence detector or MS have been reported. However, the limit of detection of melatonin using these analytical methods are around 5 fmol/injection, and a 1 - 5 mL blood sample is thus needed for the determination of melatonin. Therefore, a more sensitive and selective analytical method is highly required in order to determine endogenous melatonin in a small drop (100 μL) of plasma/serum. In the present method, the detected compound is 6-MOQMA, produced by the oxidative reaction of melatonin. The molar absorptivity and fluorescence quantum yield of this compound are 46300 [L mol⁻¹ cm⁻¹] and 0.31 (excitation at 245 nm), respectively. The fluorescence intensity of 6-MOQMA is 6.8 times higher than that of melatonin, and the highly sensitive determination of melatonin could also be carried out due to the large shift between the excitation wavelength (245 nm) and the emission wavelength (380 nm). Furthermore, the newly developed fully-automated on-line oxidation column-switching HPLC system consists of microbore-columns of Proteonavi (1.0 mm × 75 mm) for the purification of melatonin, Asahipak ODP 40-1T (1.0 mm × 35 mm) for the concentration of 6-MOQMA and CAPCELL PAK C18 IF (1.0 mm × 250 mm) for the final determination of 6-MOQMA. The lower limit of detection of melatonin using the present method is 200 amol/injection and 10 - 100 times more sensitive than those obtained by the already reported methods. In addition, the newly developed system is fully-automated, and reproducible results could be easily obtained without contamination.

Determination of endogenous melatonin in human plasma and saliva

As the application of the present fully-automated on-line oxidation column-switching HPLC system, endogenous melatonin values in human plasma and saliva were determined. For both the plasma and saliva, the endogenous melatonin was analyzed during the diurnal period and nocturnal period. Concerning the purification column used for the first step fractionation of melatonin, many interfering peaks were observed in all of the samples, and peak for melatonin could not be distinguished. On the other hand, a clear 6-MOQMA peak was observed on the analytical column after the purification and oxidation of melatonin. Figure 6 shows chromatograms of 6-MOQMA derived from melatonin in human plasma (blood was collected at 12:30 during the diurnal period, and 00:30 during the nocturnal period). Endogenous melatonin in human plasma showed a clear circadian rhythm; the concentration in human plasma was 8.1 fmol mL⁻¹ in the diurnal period, and 59.7 fmol mL⁻¹ in the nocturnal period. Figure 7 shows chromatograms of 6-MOQMA derived from melatonin in human saliva (samples were collected at 12:30 and 00:30).
Endogenous melatonin in human saliva also showed a clear circadian rhythm; the concentrations were 1.7 fmol mL$^{-1}$ in the diurnal period and 23.0 fmol mL$^{-1}$ in the nocturnal period. The amounts of melatonin in human saliva were approximately 40% of those in the plasma.

For the determination of endogenous melatonin in human plasma, various analytical methods, such as RIA, GC-MS, HPLC-ECD and HPLC-FD, have been reported. The reported amounts of endogenous melatonin in human plasma are 5 – 500 fmol mL$^{-1}$. In the present investigation, the value of melatonin was 8.1 fmol mL$^{-1}$ in the diurnal period and 59.7 fmol mL$^{-1}$ in the nocturnal period; these values were consistent with those reported in the literature. The already reported analytical methods require 1 – 5 mL of human plasma, because the concentration of melatonin in human plasma is in the range of 5 – 10 fmol mL$^{-1}$ during the diurnal period. On the other hand, the newly developed fully-automated on-line oxidation column-switching HPLC system enables the determination of melatonin using only 100 μL of human plasma, even during the diurnal period. Therefore, based on the present method, a less invasive blood collection technique could be used to determine the melatonin rhythm in patients.

Saliva is also a non-invasive clinical sample. However, collecting a few mL of saliva is practically difficult, and a
sensitive analytical method is also required. For the
determination of endogenous melatonin in human saliva, RIA,20
ELISA28 and HPLC-MS/MS30 methods have been previously
reported. The reported amounts of melatonin in human saliva
are 15 - 150 fmol mL⁻¹ during the nocturnal period, and the
values are 30 - 40% of those in the plasma.35,36 In the present
investigation, we have determined the endogenous melatonin in
human saliva; the amount was 1.7 fmol mL⁻¹ in the diurnal
period and 23.0 fmol mL⁻¹ in the nocturnal period. The amount
of melatonin in human plasma was 8.1 fmol mL⁻¹ in the diurnal
period and 59.7 fmol mL⁻¹ in the nocturnal period, therefore,
the amounts of melatonin in human saliva determined in the
present study were approximately 40% of those in the plasma.
The results obtained in the present study are consistent with
those reported in the literature. Concerning the amount of
saliva, only 300 μL of saliva was used in the present study. It is
much easier to collect 300 μL of saliva than collecting 3 mL of
saliva, indicating that the newly-developed HPLC system is
useful for the analysis of melatonin in clinical samples.

Conclusions

In the present investigation, we have established a fully-
automated on-line oxidation column-switching HPLC system
for the determination of melatonin. The HPLC system was validated, and endogenous melatonin in a small drop (100 μL)
of human plasma was successfully determined. Endogenous melatonin in a small volume (300 μL) of human saliva could also be determined. The melatonin rhythm of each patient is expected to be clarified for the diagnosis and treatment of rhythm disorders, and also for clinical use in chronotherapy. The present HPLC system is a useful tool for monitoring endogenous melatonin in small volumes of human physiological fluids, and the clinical applications are currently in progress.

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References

3. M. L. Dubocovich and M. Markowska, Endocrine, 2005,
27, 101.
4. E. Savaskan, G. Olivier, F. Meier, L. Brydon, R. Jockers,
R. Ravid, A. Wizé-Justice, and F. Müller-Spahn, J. Pineal
5. E. Savaskan, M. A. Ayoub, R. Ravid, D. Angeloni, F.
Fraschini, F. Meier, A. Eckert, F. Müller-Spahn, and R.
6. J. Scher, E. Wankiewicz, G. M. Brown, and H. Fujieda,
7. C. Ekmekcioglu, P. Haslmaier, C. Philipp, M. R. Mehrabi,
H. D. Glogar, M. Grimm, T. Thalhammer, and W. Marktl,
8. C. Ekmekcioglu, T. Thalhammer, S. Humpelet, M. R.
Mehrabi, H. D. Glogar, T. Hölzenbein, O. Markovic, V. J.
Leibetseder, G. Strauss-Blasche, and W. Marktl, J. Pineal
288.
11. D. R. Weaver and S. M. Reppert, Neuroreport, 1996, 8,
109.
12. B. Clausstrat, M. Geoffriau, J. Brun, and G. Chazot,
13. I. Papaioannou, G. L. Twigg, M. Kemp, M. Roughton, J.
Hooper, M. J. Morrell, and M. I. Polkey, Sleep Med., 2012, 
13, 167.
19, 544.
17. E. A. de Almeida, P. D. Mascio, T. Harumi, D. W. Spence,
A. Moscovitch, R. Hardeland, D. P. Cardinalli, G. M. Brown,
27, 879.
18. S. Fraser, P. Cowen, M. Franklin, C. Franey, and J. Arendt,
20. A. Miles, D. Philbrick, S. F. Tidmarsh, and D. M. Shaw,
22. B. W. Wilson, W. Snedden, R. E. Silman, I. Smith, and P.
24. G. Simonin, L. Bru, E. Lelièvre, J.-P. Jeannot, N. Bromet,
1999, 21, 591.
Biochem., 1992, 205, 300.
26. V. Rizzo, C. Porta, M. Moroni, E. Scoglio, and R. Moratti,
27. M. D. Carter, M. W. Calcutt, B. A. Malow, K. L. Rose, and
28. T. Kozaki, S. Lee, T. Nishimura, T. Katsuura, and A.
2003, 794, 115.
31. F. Inuma, K. Hamase, S. Matsubayashi, M. Takahashi, M.
Biochem., 2000, 279, 106.
33. T. Tomita, K. Hamase, H. Hayashi, H. Fukuda, J. Hirano,
34. K. Hamase, J. Hirano, Y. Kosai, T. Tomita, and K. Zaitsu, J.
35. I. M. McIntyre, T. R. Norman, G. D. Burrows, and S. M.