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Estimation of time of death by quantification of melatonin in corpses

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Abstract A method for the estimation of time of death (TOD), was evaluated by measuring the melatonin (MT) content of pineal bodies (PBs), sera and urine samples from 85 cadavers. A total of 44 cadavers were investigated in Sapporo (geographical coordinates N 43° 4′, E 141° 21′) and 41 in Tokyo (N 35° 39′, E 139° 44′). MT contents were measured by radioimmunoassay (RIA) in 75 PBs, 27 sera and 14 urine samples. Exponential differences of pineal MT content were recognized between peaks in nighttime and nadirs in daytime, ranging from 0.099 to 63.2 ng/PB. Circadian rhythms were also observed for the concentrations of MT in serum (11–205 pg/ml), and in urine (7.5–137.5 pg/ml). Consequently, criteria for the TOD estimation are proposed as follows. 1) Pineal MT contents – (1) 0–0.2 ng/PB: TOD 1100–1700 hours, (2) 0.2–0.3 ng/PB: TOD 0700–2000 hours, (3) 0.3–1 ng/PB: inconclusive, (4) 1–4 ng/PB: TOD 1600–1000 hours, (5) 4–8 ng/PB: TOD 2000–0800 hours, (6) over 8 ng/PB: TOD 2000–0500 hours, 2) Serum MT concentration – (1) 0–100 pg/ml: inconclusive, (2) over 100 pg/ml: TOD 2200–0100 hours, and 3) Urinary MT concentration – (1) 0–35 pg/ml: inconclusive, (2) over 35 pg/ml: TOD 1800–0600 hours. The range of the estimation can be limited by a combination of these 3 criteria. The present method can be combined with other methods for estimating the TOD to decrease the range.

Key words Estimation of time of death · Melatonin Radioimmunoassay · Pineal body · Circadian rhythm

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Zusammenfassung Eine Methode zur Bestimmung der Todeszeit (TZ) wurde evaluiert, indem der Melatonin-Gehalt der Epiphysen, Serum und Urin-Proben von 85 Leichen gemessen wurde. Insgesamt wurden 44 Leichen in Sapporo (geographische Koordinaten N 43° 4′, E 141° 21′) und 41 Leichen in Tokio (N 35° 39′, E 139° 44′) untersucht. Die Melatonin-Gehalte wurden mit Hilfe des Radioimmunoassays (RIA) in 85 Epiphysen, 27 Seren und 14 Urinproben untersucht. Exponentielle Differenzen des epiphysären Melatonin-Gehalts wurden zwischen den nächtlichen Spitzenwerten und den „Nadirs" während der Tageszeit beobachtet. Diese variierten zwischen 0,099 und 63,2 ng/Epiphyse. Zirkadian-Rhythmen wurden ebenfalls beobachtet für die Konzentrationen von Melatonin im Serum (11–205 pg/ml) und in Urin (7,5–137,5 pg/ml). Folglich werden die Kriterien für die Bestimmung der TZ wie folgt vorgeschlagen: 1) Epiphysäre Melatonin-Gehalte – (1) 0–0,2 ng/Epiphyse: TZ 1100–1700 Uhr, (2) 0,2–0,3 ng/Epiphyse: TZ 0700–2000 Uhr, (3) 0.3–1 ng/Epiphyse: unentschieden, (4) 1–4 ng/Epiphyse: TZ 1600–1000 Uhr, (5) 4–8 ng/Epiphyse: TZ 2000–0800 Uhr, (6) über 8 ng/Epiphyse: TZ 2000–0500 Uhr; 2) Serum-Melatonin-Gehalte – (1) 0–100 pg/ml: unentschieden, (2) über 100 pg/ml: TZ 2200–0100 Uhr und 3) Urin-Melatonin-Gehalte – (1) 0–35 pg/ml: unentschieden, (2) über 35 pg/ml: TZ 1800–0600 Uhr. Die Streubreite der Bestimmung kann durch eine Kombination dieser 3 Kriterien eingeengt werden. Die vorliegende Methode kann mit anderen Methoden zur Bestimmung der Todeszeit kombiniert werden, um deren Variationsbreite zu verringern.

Schlüsselwörter Bestimmung der Todeszeit · Melatonin Radioimmunoassay · Epiphyse · Zirkadian-Rhythmus

Introduction

Estimation of the postmortem interval is an important factor for the forensic practice. We have examined this by observing variations in the progression of postmortem changes of melatonin (MT).

Biological rhythms have been studied in the field of physiology and the existence of biological clocks have been demonstrated. Most animate things have biological clocks and live according to circadian rhythms. This phenomenon could be applied to forensic medicine, in order to estimate the time of death (TOD).

One substance which shows a biological rhythm is melatonin, which is a hormone secreted from the pineal body (PB). The synthesis of MT increases vastly in nighttime and decreases in daytime [1]. It is not influenced by environmental factors except light and, in the case of humans, it is only influenced by bright light [2]. Therefore, measurement of the MT level in corpses could define TOD with clock hour or at least could distinguish between daytime and nighttime [3–5].

To test this hypothesis, we measured the MT content in PBs, sera and urines from cadavers with known time of death. We investigated changes in MT levels with respect to postmortem intervals and differences by sex, age and season. We also propose a scheme for the application of the circadian rhythm of MT to the estimation of TOD.

Materials and methods

Samples were taken from 44 corpses autopsied in the Department of Legal Medicine, Hokkaido University, School of Medicine from January 1989 to November 1991, consisting of 24 males (4–90 years) and 20 females (4–76 years). Samples were also taken from 41 corpses in the Department of Forensic Medicine, Faculty of Medicine, University of Tokyo and in Tokyo Medical Examiner's Office from May to October 1991 consisting of 28 males (21–88 years) and 13 females (21–94 years). We investigated the MT contents in PBs, sera and urines in these 85 corpses of which 48 died from external causes (cerebral injury 8, other injury 20, asphyxiation 12, poisoning 6, hypothermia 2), 33 died of intrinsic causes and the cause of death was unknown in 4 cases. Out of these, PBs (75 samples), blood (27 samples) and urine (14 samples) were taken from selected cases because the TOD was known. Samples were frozen and stored at –70°C. PBs were removed from their surrounding tissues and weighed after natural thawing at room temperature (RT).

Measurement of MT by Radioimmunoassay (RIA)

MT was measured using a Melatonin Radioimmunoassay kit (ITS Production B. V., Netherlands) with minor modifications to the manufacturer's instructions as follows: each PB was homogenized in 2.0 ml of 0.1 N HCl for 1 min on ice. The homogenate was divided equally by weight into 2 centrifuge tubes. To one of the tubes 50 µl of a solution containing approximately 2700 cpm of ^{125}I-MT was added as a tracer. To the other tube 50 µl of 0.1 N HCl was added for the assay. Each tube was Vortexed for 1 min and supernatants were collected after centrifugation at 2000 × *g* for 10 min. To each tube 2 vol of 0.05 M phosphate buffered saline (PBS) (pH 7.4) was added to the supernatant, mixed, and neutralized with 2.5 N NaOH. An aliquot of 0.5 ml was transferred to another centrifuge tube and mixed with 3.0 ml of diethyl ether for 1 min.

Following centrifugation for 5 min at 2000 × *g*, the water phase was frozen at –20°C for 30 min and the ether layer was transferred to another tube. The extract was dried in a stream of nitrogen, and redissolved with 0.5 ml of assay buffer (containing bovine serum albumin, pH 7.4) by agitation, or stored at –15°C if not assayed immediately.

The "MT standard" in the kit was diluted with assay buffer to give, 640, 320, 160, 80, 40, 20, 10, 5, and 0 pg/ml. An aliquot of 200 µl of each standard or extracted sample was pipetted into tubes, 100 µl of antiserum was added, stirred and incubated for 1 h at 37°C. For measuring non-specific reaction, 300 µl of standard buffer was similarly prepared.

To each tube 100 µl of tracer was added, incubated for 18 h at 4°C after stirring followed by 100 µl of separation reagent with stirring. After incubation for 20–30 min, 1.0 ml of distilled water was added and agitated. Following centrifugation for 15 min at 2000 × *g* at RT, the precipitate was measured using a gamma-counter (Aloka ARC-605, Tokyo, Japan).

Simulation studies for postmortem change of MT contents

Experiments were carried out to investigate the influence of postmortem interval on the results.

We took 5 g of human cerebral cortex, as a model for the pineal body, added 20 ml of distilled water and 5 ng of MT (Aldrich Chemical Co., Milwaukee, USA) dissolved in 50 µl of ethanol. The mixture was homogenized and 1.0 ml of homogenate was placed in 9 tubes and centrifuged for 10 min at 2000 × *g*. After removing the supernatants by aspiration, we sealed the tubes with Parafilm and kept them in the dark at RT for periods of 0.5 h, 1 h, 3 h, 6 h, 12 h, 24 h, 2 d, 4 d and 7 d. Similarly, 5.5 ng of MT was added to 5.5 ml of blood or urine samples and divided into 9 tubes. MT contents of these simulations were measured by RIA.

Results

Measuring method of MT

The standard curve was generated using MT standards prepared at the time of each assay.

We subtracted the average of the non-specifically bound (NSB) counts from the average counts of other duplicate tubes to obtain a net corrected cpm, and calculated the percentage of maximal binding by dividing the corrected cpm of the zero standard (B_0) by the average cpm

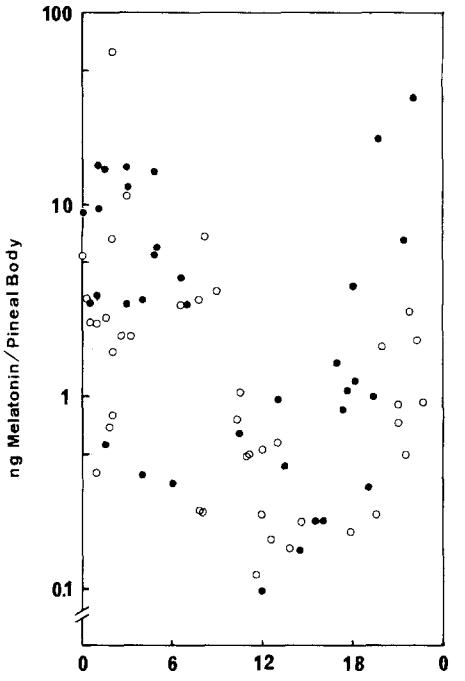

Fig. 1 The amount of MT in each pineal body of cadavers and the time of death (● = obtained in Sapporo; ○ = obtained in Tokyo)

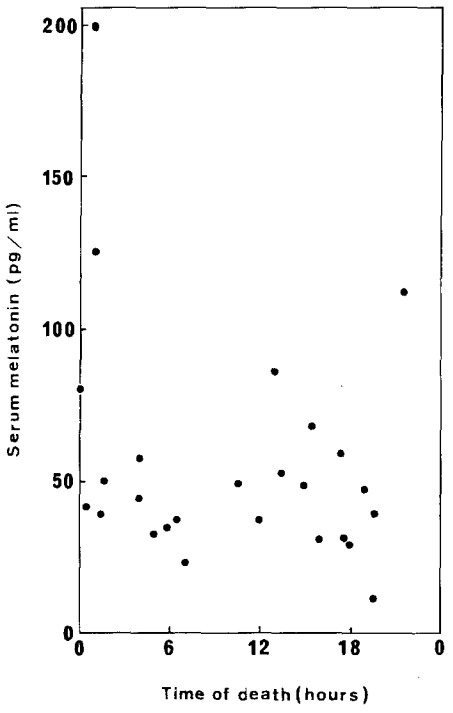

Fig. 2 The amount of MT in serum of cadavers and the time of death

of the total counts. We calculated the percentage B/B_0 for each standard pair, similarly. NSB/total counts were given a 3.9% by the manufacturers, whereas our results showed 5.3–7.8% (mean 6.6%).

The volume of homogenate recovered after centrifugation was 0.74–1.08 ml (mean 0.88 ml = 88%). In the case of PBs the mean recovery ratio of MT was 55.8% (32.4–79.8%), 77.6% (44.3–98.4%) in sera and 80.9% (40.0–100.0%) in urine.

Relationship between time of death (TOD) and MT contents

We plotted the logarithmic amounts of MT for each PB (ng/PB) on the ordinate against the TOD on the abscissa. MT contents had a tendency to be high during the nighttime and low during the daytime as shown in Fig.1.

The MT value ranged from 0.1 to 1 ng at noon and 2–10 ng at midnight. The minimum value was 0.099 ng/PB (1200 hours) and the maximum was 63.16 ng/PB (0200 hours).

The relationship between serum MT and TOD is shown in Fig.2. The deviation of MT contents in the nighttime was large (39–205 pg/ml), but small in the daytime (11–86.5 pg/ml). The circadian rhythm was recognized with a minimum of 11 pg/ml (1940 hours) and a maximum of 205 pg/ml (0100 hours).

Similarly to PB and serum cases, there appeared to be a circadian rhythm of MT in urine (Fig.3) with a minimum of 7.8 pg/ml (1220 hours) and a maximum of 137.5 pg/ml (2100 hours).

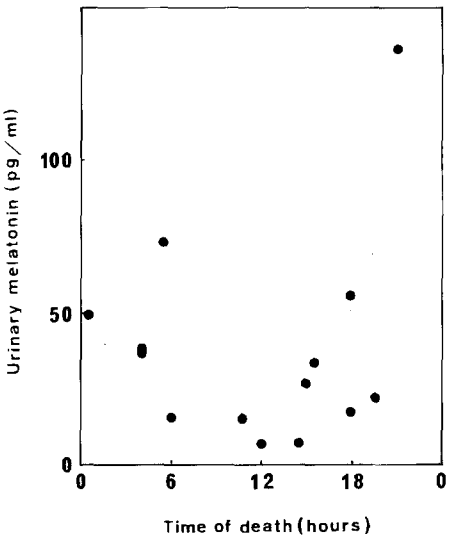

Fig. 3 The amount of MT in urine of cadavers and the time of death

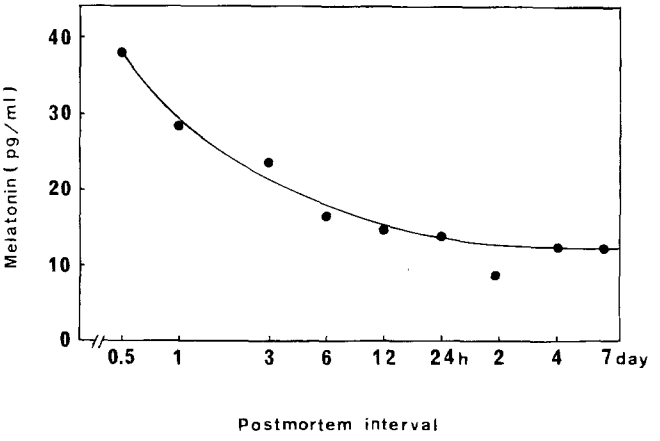

Fig. 4 Degradation of MT by postmortem intervals using human cerebral cortex as a model for the pineal body

Simulation studies for postmortem changes

The rate of degradation of cerebral MT is shown in Fig.4. The amount of MT gradually decreased over a 24 h period, and remained at the level of about one third of the original amount after 24 h and later.

There was no obvious degradation in serum MT and urinary MT.

Discussion

Quantification and recovery ratio of MT

In this study, extraction by diethyl ether was applied following the instruction manual of the kit. Since the recovery ratio of MT varied in each pineal body sample, the ratio was tested in each quantification. The extraction recovery of MT in PBs was reported to be 70% by petroleum ether [6], whereas that in the present extraction with

diethyl ether was inferior. As an extraction system with low recovery efficiency, which requires larger amounts of specimens, causes greater error in assay, and discourages quantification of MT in other tissues, an extraction protocol with higher recovery would be necessary.

Relationship between TOD and MT contents

Greiner and Chan [6] reported that a circadian rhythm in human PBs was recognized at the range of 0–71.1 ng/PB (n = 22), and its value was high during the nighttime but low during the daytime. But Beck et al. [7] stated that there were no differences in MT contents between nighttime and daytime ranging from 0.86 to 391.5 ng/PB (we converted pmoles/g reported to ng/PB: n = 11). In our studies the MT contents ranged from 0.099 to 63.2 ng/PB which was similar to the result of Greiner and Chan [6].

MT circadian rhythms have also been recognized in rats, chickens and quails with MT ranges 0.5–6.8, 0.6–11.1 and 0.5–3.2 ng/PB, respectively [8].

Previous reports [7, 9] have indicated large individual differences in humans with regard to pineal MT contents.

As the number of our samples (n = 75) was larger than those in previous reports, this would appear to exclude influences caused by individual differences.

Although relationships between MT contents and various conditions have been reported, we investigated only the relationship between MT contents/PB and TOD without considering any other conditions and found a clear circadian rhythm as shown in Fig.1.

Reiter [10] reported that there were differences in serum MT among species of mammals. The pattern of nocturnal increase in MT contents seemed to be specific in various mammals. Three different types of nocturnal MT rhythm were identified.

In type I, MT levels are high only for a brief interval in the latter half of the dark period. MT production rises rapidly, reaches a peak of short duration and begins to decline before or at the end of dark period. This pattern of pineal MT production is seen in Syrian hamsters and house mice.

The type II pineal MT rhythm exists in a number of mammals. In these species MT production begins to increase at or shortly after the onset of darkness; the values continue to rise gradually to reach a peak near the middle of the dark phase. MT levels then begin to drop to reach daytime values at about sunrise. This rhythm seems to be most common and is typically found in squirrels, Turkish hamsters and humans.

In animals with type III rhythm, pineal MT values increase rapidly after dark to reach a plateau which is maintained for the duration of the dark phase. Either shortly before or at the beginning of the light period MT values drop to low levels. This type of rhythm is typical of the pineal gland of domestic cats and sheep, etc.

From the data obtained in the present experiments, human pineal MT contents increase immediately and exponentially at the onset of darkness, the peak is maintained for the middle of the dark period, and decrease rapidly at dawn. This pattern is a mixture of the types II and III.

Table 1 The diurnal variation of serum melatonin reported in the literature

Investigators	Diurnal variation of MT (pg/ml)[a]	References
Pelham et al.	0– 55	[11]
Arendt	10–150	[12]
Arendt et al.	0– 75	[13]
Iguchi et al.	10– 80	[14]
Iguchi et al.	0–120	[15]
Ehrenkranz et al.	10– 90	[16]
Tamarkin	18– 65	[17]
Arendt et al.	0– 85	[18]
Berga et al.	10–120	[19]
Sturner et al.	0– 97.9	[20]
Petterborg et al.	0–104	[21]
Smith et al.	68.6±24.4[b] (female) 64.9±15.2[b] (male)	[22]
Karasek et al.	18.6–95.1	[23]

[a] In some cases figures are estimated from graphs, or recalculated from different units
[b] Only in nighttime

From the results shown in Fig.1, we can propose the following criteria for an estimation of TOD from the level of pineal MT contents/PB.

(1) 0–0.2 ng/PB: TOD 1100–1700 hours
(2) 0.2–0.3 ng/PB: TOD 0700–2000 hours
(3) 0.3–1 ng/PB: inconclusive
(4) 1–4 ng/PB: TOD 1600-1000 hours
(5) 4–8 ng/PB: TOD 2000–0800 hours
(6) over 8 ng/PB: TOD 2000–0500 hours

Stanley and Brown [9] stated that pineal MT contents in suicides was lower than those in persons who died from gunshots, traffic accidents and myocardial infarction. Their data showed a wide variation which was similar to our data.

Research into human MT contents has been carried out mainly on serum samples with levels ranging from 0–20 pg/ml in daytime to 60–120 pg/ml in nighttime [2]. Since serum MT is derived from the PB, it is at a much lower level. As MT contents are generally very low, highly sensitive and elaborate measuring methods such as RIA have been developed and utilized.

The diurnal variation of serum MT between daytime and nighttime [11–23] is also much smaller than that of PB (Table 1).

Large variation is recognized in serum MT concentrations as indicated in Fig.2 and large individual differences have also been observed, especially in nighttime [24].

TOD can only be estimated between 2200 to 0100 hours when MT contents are more than 100 pg/ml (Fig.2), indicating that the measurement of pineal MT is more useful than that of serum MT.

There are 2 main problems associated with urinary MT. Firstly there is the problem of the accumulation time of MT in the urinary bladder. Wetterberg [25] found a correlation between human serum MT at 2:00 a.m and urinary MT collected in the early morning period (correlation coefficient 0.89). This does not always mean that the amount of uri-

nary MT synthesized in the body at a certain time reflects the MT level at the time of taking urine, i.e., the amount of urinary MT does not always indicate the MT level at the time of death but the accumulated amount between the last urination and death. For example, if a person died at noon without urinating during the night, the amount of urinary MT in the daytime could be misinterpreted as the MT level at midnight, leading to a false estimation of the TOD.

Secondly there is the problem of MT metabolism. MT in pinealocytes of the PB is produced from N-acetyl-serotonin (NAS) by hydroxyindole-o-methyl transferase, then rapidly released into the blood [26]. It is metabolized in the liver to conjugates of 6-hydroxymelatonin (6-OHMT) [27], i.e. 6-sulphatoxymelatonin (aMT6s) [28] and glucuronide 6-OHMT [29] and in the brain primarily to N-acetyl-N-formyl-5-methoxy kynurenamine and secondly to N-acetyl-5-methoxy kynurenamine [30]. These metabolites are excreted through the kidney into the urine [31].

In previous studies concerning the metabolic fate of MT, various metabolites of MT have been identified in urine [32, 33]. In rats and rabbits, aMT6s (55–80%) and glucuronide 6-OHMT (5–30%) were found to be excreted into urine. Also NAS, which is the nearest precursor of MT and excreted as glucuronide and sulfate conjugates, is a minor metabolite of MT (< 2% of urinary metabolites), and the proportion of NAS to 6-OHMT ranged from 9 to 21%, with a mean of 15% in humans [34]. Free 6-OHMT (< 2% of urinary metabolites) in rat urine, 1–4% in humans, 5-methoxy-3-indoleacetic acid (< 2%), and cyclic 2-hydroxy-MT have also been reported [33].

It is concluded that the major metabolic products of MT are 6-OHMT conjugates, especially aMT6s, the levels of which can be significant [35]. It is possible that aMT6s was measured instead of MT in urine because the RIA kit has very weak cross-reactivities with 6-OHMT (1.00%) and NAS (0.02%). According to Bojkowski and Arendt [36], the amount of aMT6s excreted in one day ranged between 4.04 and 10.8 μg indicating a concentration range of 2693–10790 pg/ml for a daily urinary volume range of 1000–1500 ml. Using the above ratio "55–80%" the volume of aMT6s in daily urine is calculated to be 1481–8632 pg/ml. The cross-reactivity of 6-OHMT in this RIA kit is 1% and if it had cross-reacted completely, the calculated value would be 14.8–86.3 pg/ml. This result is similar to our data for the MT range in urine. Similarly, Tetsuo et al. [37] reported that the total amount excreted was 12.9 ± 0.6 (SEM) μg/day for boys, 15.9 ± 0.8 for girls, and 11.4 ± 0.75 for adults and corresponds to the values found in our results. This would imply that we have measured the amounts of aMT6s instead of MT in urine.

As MT in liver is rapidly metabolized, it is suggested that the level of urinary 6-OHMT reflects that of serum MT at TOD, excluding urination problems caused by head injuries, benign prostatic hypertrophy, etc. Therefore it is supposed that the estimated amounts of MT in urine would appear to be higher than the actual levels and not lower.

A close linear relationship between urinary and serum MT levels (Fig.5) could not be found. Bojkowski et al. [35] reported on the relation between urinary aMT6s and plasma MT (r = 0.75).

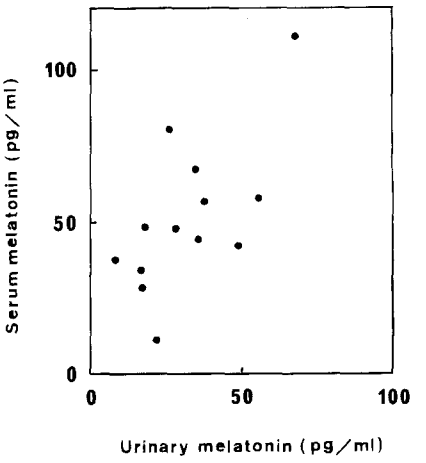

Fig. 5 Urinary and serum MT

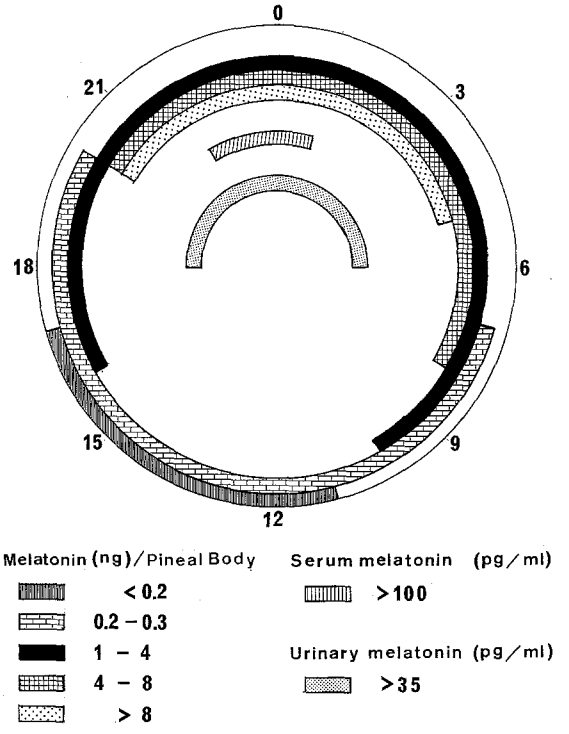

Fig. 6 Criteria for the estimation of time of death (24 hour clock indication). PB; 0.3–1 ng/PB inconclusive for death time estimation, Serum: < 100 pg/ml inconclusive for death time estimation, Urine; < 35 pg/ml inconclusive for death time estimation

The criteria for estimation of TOD from urinary MT is suggested as follows (Fig.3):

(1) 0–35 pg/ml: inconclusive
(2) over 35 pg/ml: TOD 1800–0600 hours

Apart from the 2 problems mentioned above, the estimation of TOD by measuring urinary MT would be useful in medicolegal practice.

If the values for MT levels of PB, serum and urine are combined, TOD could be estimated to a narrower margin (Fig.6). For example, if the ranges of MT contents showed 0.2–0.3 mg/PB (0700–2000 hours) and over 35

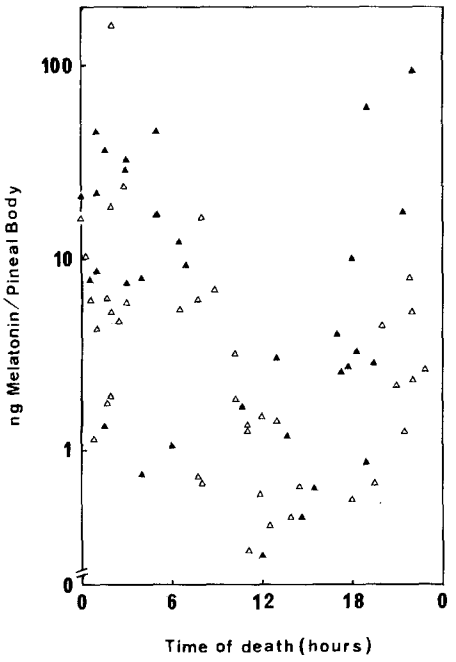

Fig. 7 The amount of MT in each pineal body of cadavers and the time of death by correlation of MT levels with postmortem intervals (▲ = obtained in Sapporo; △ = obtained in Tokyo)

pg/ml (1800–0600 hours) in urine, the TOD is estimated to be 1800–2000 hours, which is narrower than the separately estimated periods.

We will discuss TOD of 4 cases in which MT contents were measured without knowing the exact TOD.

Case 1: TOD was estimated to be 2000–0800 hours by postmortem appearances. Pineal MT content was over 4 ng/PB (2000–0800 hours), serum MT content was 91 pg/ml (0100–2200 hours), but urine was not investigated. The combined range gives the time of death between 0100–0800 hours, and the estimation time can be reduced by 5 h.

Case 2: Pineal content was 0.455 ng/PB which is inconclusive for the estimation of TOD. In this case, only postmortem appearances can be used for the estimation.

Case 3: Pineal MT content was 9.5 ng/PB (2000–0500 hours). Neither serum nor urinary MT content was measured. The TOD range estimated by postmortem appearances was 2300–0800 hours. The estimation time can be shortened by 3 h.

Case 4: Pineal MT content 78 ng/PB (2000–0500 hours), serum MT content 111 pg/ml (2200–0100 hours) and urinary MT content 67.5 pg/ml (1800–0600 hours) giving a combined range for TOD of 2200–0100 hours. There is a difference between this result and the estimated range by postmortem appearances (2300–0700 hours) and the combined range (2300–0100 hours) is narrowed by 6 h. The useful estimation could be made that death occurred with high probability at midnight.

Simulation studies for postmortem changes

We should consider the influence of postmortem changes of MT, because our samples had various postmortem intervals (3.5–48 h). The revised rates given in Fig.4 were calculated from the values in Fig.1 by dividing the MT level after 0.5 h (38 pg/ml) by the actual value after each corresponding postmortem interval. For example, the revised rate is calculated to be 1.73 for the MT concentration 22 pg/ml after a 3 h postmortem interval.

In this way using Figs.1 and 4, we plotted revised values as in Fig.7. According to this result, the criteria of TOD are as follows:

(1) 0–0.5 ng/PB: TOD 1100–1500 hours
(2) 0.5–1.0 ng/PB: TOD 0500–1900 hours
(3) 1–2 ng/PB: inconclusive
(4) 2–5 ng/PB: TOD 1000–0100 hours
(5) 5–15 ng/PB: TOD 1800–0900 hours
(6) over 15 ng/PB: TOD 1900–0500 hours

The ranges are different from those of the non-revised criteria. We cannot exactly describe the anticipated result when using PB tissue, because cerebral cortex was used in this simulation instead of PB. Therefore both estimations should be carried out. No references could be found in the literature about postmortem changes of pineal MT contents, so further studies are required to clarify this. Sturner et al. [20] reported that there was no significant correlation between the amount of serum MT and postmortem interval. Kawata [38] stated that cerebral enzymes acting on catecholamines were very stable postmortem and therefore amines decreased in the course of postmortem changes. It is therefore possible that the biological organization of oscillating circadian rhythms functions even after death. But no affirmative result supporting this idea was obtained through our experiments, because distinct differences were observed between MT concentrations in nighttime and daytime regardless of postmortem intervals.

As no changes in postmortem MT contents were recognized in serum and urine, the criteria for estimation could be used without revision.

Relationships with various factors

Relationships with sex, age, season and disease

The analysis of postmortem brain data is difficult. Great care must be taken to exclude false correlations owing to the heterogeneity of the material with respect to various factors such as age, sex, disease, agonal state and postmortem delay [39].

With regard to a sexual difference of MT, it has been reported that no significant differences could be recognized in whole blood samples from children of different sexes [20], but no references could be found regarding pineal MT.

It has been reported that very old individuals (84–86 years old) have markedly depressed MT levels in blood, especially during the daily dark period [14]. As men-

Table 2 Pineal melatonin contents in nighttime and daytime by age with respect to sex

Range of age (years)	Time of death		Male			Female		
			n	Age (years)	Pineal MT[a] (ng/PB[b])	*n*	Age (years)	Pineal MT (ng/PB)
All ages	Night and day	Mean	48	47.4	4.48	29	52.1	7.21
		S.D.		20.1	9.86		20.7	15.27
	Night[c]	Mean	26	49.1	7.50	21	50.0	9.78
		S.D.		16.8	12.70		22.7	17.35
	Day[d]	Mean	22	45.3	0.92	8	58.3	0.35
		S.D.		24.1	1.07		13.1	0.25
0–20	Night and day	Mean	2	4.5	2.36	1	4.0	5.64
		S.D.		–	–		–	–
	Night	Mean		–	–	1	4.0	5.64
		S.D.		–	–		–	–
	Day	Mean	2	4.5	2.36		–	–
		S.D.		–	–		–	–
20–40	Night and day	Mean	11	29.3	2.65	8	31.0	5.13
		S.D.		6.1	3.62		6.7	5.47
	Night	Mean	7	28.1	3.81	7	30.0	5.78
		S.D.		5.4	4.18		6.5	5.62
	Day	Mean	4	31.3	0.64	1	38.0	0.91
		S.D.		7.5	0.40		–	–
40–60	Night and day	Mean	22	48.7	4.89	9	51.6	2.10
		S.D.		5.2	6.07		5.6	2.80
	Night	Mean	13	50.1	7.57	6	51.5	3.00
		S.D.		4.6	6.67		5.7	3.14
	Day	Mean	9	46.7	1.03	3	51.7	0.39
		S.D.		5.7	1.26		6.7	0.17
60–	Night and day	Mean	13	70.7	5.67	11	72.5	13.31
		S.D.		9.2	17.3		9.8	23.94
	Night	Mean	6	71.5	11.66	7	74.9	20.67
		S.D.		10.2	25.27		10.6	28.00
	Day	Mean	7	69.8	0.53	4	68.3	0.38
		S.D.		9.1	0.43		7.6	0.31

[a] MT: melatonin
[b] PB: pineal body
[c] Night: 2000–0800 hours
[d] Day: 0800–2000 hours

tioned by Nair et al. [40], the peak of plasma MT level of young men was higher than that of elderly men, and a study of the circadian rhythm of plasma MT might prove to be a useful index of the aging process, and may assist the estimation of age. During the dark period, the increase of pineal MT in old (18-month-old) Syrian hamsters was reported to be less than that in young animals [41].

With regard to seasonal differences, Arendt et al. [42] reported that serum MT was low in spring and fall, and high in winter and summer. Wetterberg [25] also described that the weight of PBs showed an annual variation with the highest weights in March and the lowest in July for males and highest in January and the lowest in May for females.

The following references were found regarding influences of diseases: serum level of MT in patients with liver cirrhosis was significantly elevated compared to those in healthy subjects [15]; whole blood MT levels were significantly lower in SIDS (sudden infant death syndrome) infants than in the non-SIDS infants [20]; the MT levels during the early hours of the night appeared to be higher in patients with seasonal affective disorders than in the controls [43].

An analysis of our data regarding the above factors can be seen in Tables 2–5.

Sexual difference and difference in age

According to Table 2, the amounts of MT in the pineal body (MT/PB) are likely to be larger in females than in males without considering any other factors. Furthermore, the amounts are larger in females below 40 years of age and over 60 years but smaller than males in the range between 40 and 60 years old. A comparison of the time of death in nighttime (2000–0800 hours) and in daytime (0800–2000 hours) shows that MT contents in females tend to be slightly larger than in males in nighttime, but are smaller in females in daytime. The levels of MT/PB are higher in females 20–40 years old, but lower in females 40–60 years old.

Table 3 Serum melatonin in nighttime and daytime with respect to sex

Time of death	Male			Female		
	n	Age (years)	Serum melatonin (pg/ml)	*n*	Age (years)	Serum melatonin (pg/ml)
Night and day	18	42.9 ± 21.5[a]	46.2 ± 20.4	11	49.0 ± 15.2	94.4 ± 63.1
Night[b]	9	45.1 ± 13.6	41.3 ± 21.9	8	49.2 ± 17.0	115.3 ± 62.0
Day[c]	9	39.1 ± 26.5	51.1 ± 18.6	3	48.3 ± 12.1	38.7 ± 8.0

[a] Mean ± S.D.
[b] Night: 2000–0800 hours
[c] Day: 0800–2000 hours

Table 4 Urinary melatonin in nighttime and daytime with respect to sex

Time of death	Male			Female		
	n	Age (years)	Urinary melatonin (pg/ml)	*n*	Age (years)	Urinary melatonin (pg/ml)
Night and day	11	37.0 ± 25.0[a]	44.0 ± 36.1	5	59.2 ± 10.8	42.6 ± 27.9
Night[b]	6	32.2 ± 15.5	56.1 ± 45.3	4	58.5 ± 12.4	51.3 ± 23.2
Day[c]	5	44.2 ± 36.7	30.5 ± 15.8	1	62.0	7.8

[a] Mean ± S.D.
[b] Night: 2000–0800 hours
[c] Day: 0800–2000 hours

Table 5 Seasonal differences of pineal melatonin contents in nighttime and daytime

Seasons	Time of death	*n*	Age (years)	Pineal MT[a] (ng/PB[b])
Winter[c]	Night and day	10	45.5 ± 18.0[i]	5.88 ± 5.73
	Night[g]	8	43.7 ± 19.3	7.15 ± 5.74
	Day[h]	2	52.5	0.80
Spring[d]	Night and day	9	41.5 ± 21.2	7.37 ± 11.45
	Night	5	49.2 ± 18.8	12.61 ± 13.60
	Day	4	28.7 ± 21.6	0.82 ± 0.33
Summer[e]	Night and day	30	49.7 ± 17.4	1.60 ± 1.94
	Night	16	47.6 ± 15.3	2.36 ± 2.23
	Day	14	52.1 ± 20.0	0.73 ± 1.07
Fall[f]	Night and day	29	52.4 ± 21.1	9.00 ± 18.03
	Night	19	53.6 ± 23.1	11.95 ± 21.31
	Day	10	50.1 ± 17.3	3.12 ± 6.94

[a] MT: melatonin
[b] PB: pineal body
[c] Winter: December–February
[d] Spring: March–May
[e] Summer: June–August
[f] Fall: September–November
[g] Night: 2000–0800 hours
[h] Day: 0800–2000 hours
[i] Mean ± S.D.

For serum MT (Table 3), a difference between the sexes can be recognized that MT of females is higher in nighttime than in daytime, and in males there was no difference between nighttime and daytime. Therefore females have a different pattern in the circadian rhythm of serum MT than males.

Furthermore, MT contents in females are more than those in males. These conclusions are different from those reported by Iguchi et al. [14] and Sturner et al. [20].

For urinary MT contents (Table 4), only small differences could be recognized by sex and by age.

Seasonal differences

Weights of PBs decreased in the order of spring, fall, summer and winter (Table 5). As the difference among the seasons is small, there might be no seasonal variation for PB weight, although Wetterberg [25] reported the existence of such a variation.

The amounts of MT/PB decreased in the order of fall, spring, winter and summer. These results differ from those of Arendt and Arendt et al. [12, 42] who described 2 peaks of serum MT in winter and summer.

Furthermore, there is a tendency that the amounts of MT/PB in nighttime decreased in the order of spring, fall, winter and summer, but in daytime the order was fall, spring, winter and summer.

Disease and PB calcification

We have investigated a case of cirrhosis and found that the amount of MT in PB, serum, urine was lower than average. This result is different from the report of Iguchi et al. [15] who alleged that the amount of serum MT in a case of cirrhosis was larger than that of PB.

The degree of calcification in PB tended to increase with aging. No correlations between the amount of MT/PB, or differences between the sexes could be recognized.

Zeitgeber (synchronizer)

Biological rhythms have their own natural periodicity, but are usually regulated by a Zeitgeber. Synchronising factors in human circadian rhythms are; (1) social factors, (2) environmental light-dark cycles, (3) effects of sleep-wake schedule, (4) recognition of time, (5) timing of diet, etc.

Petterborg et al. [21] reported that serum MT levels decreased significantly in humans subjected to bright light of approximately 1500 lux. Adler et al. [44] stated that the normal increase in MT in nighttime was observed in dim light (< 50 lux), whereas MT concentrations were lowered when the light conditions were under 1000 lux. They were not influenced by normal daylight but by bright light such as sunlight. Sunlight changes under various meteorological conditions, such as day and night, seasons, regions

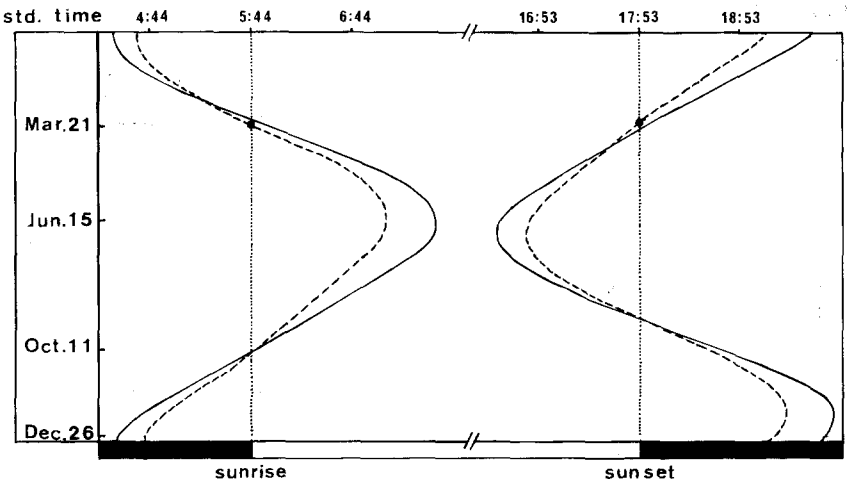

Fig. 8 Shift of the sunrise and sunset time by regional and seasonal differences. Std. time: time of the Vernal Equinox Day in Tokyo where sunrise time is at 5:44 and sunset time is at 17:53. Dashed lines represent the time of 5:44 (left) and 17:53 (right) in Tokyo (N 35° 39′, E 139° 44′); solid lines represent the time of 5:44 (left) and 17:53 (right) in Sapporo (N 43° 4′, E 141° 21′). Figures are based on Scientific Tables

(latitude, longitude and altitude) and daylight hours influenced by the weather. As we have already described differences between day and nighttime and seasonal differences, we will mention regional differences and the effects of daylight hours.

Regional differences

Samples examined in this study were obtained from Tokyo (N 35° 39′, E 139° 44′) and Sapporo (N 43° 4′, E 141° 21′). A comparison between these 2 cities shows that at the summer solstice, the sun rises 30 min earlier in Sapporo, and sets 18 min later than in Tokyo. Conversely, at the winter solstice, it rises 16 min later and sets 28 min earlier. This could lead to errors in the estimation of TOD according to MT rhythms which are synchronized by sunlight. Seasonal changes of sunrise and sunset in Tokyo and in Sapporo were made by using standard times for the Vernal Equinox in Tokyo where the sun rose at 5:44 and set at 17:53, as seen in Fig.8 [45] and the actual times were derived from this. For example, 7:00 on 5th January in Sapporo is before sunrise but in June, it is 3 h after sunrise. We might regard 7:00 (the original time) as 5:40 (the revised time) at standard time (time of the Vernal Equinox Day in Tokyo).

The means of MT contents of samples, in the same period from August to October in Tokyo and Sapporo, are listed in Table 6. Regardless whether night or day, the MT contents obtained in Sapporo seemed to be larger than those in Tokyo, and variations of MT contents in Sapporo during day and nighttime tended to be greater than in Tokyo. From these results, it seems that the higher the latitude, the larger the difference of content of pineal MT between peak and nadir. The data of Beck et al. [7] also showed a wide amplitude in Stockholm which is located at a high latitude (N 59° 21′, E 17° 57′).

Differences of daylight hours

The average daylight hours in a month in both Tokyo and Sapporo are shown in Fig.8. In Tokyo, the daylight hours in both July and September are shorter than those in August, probably because of the rainy season or typhoon.

Table 6 Regional differences of pineal melatonin between Tokyo and Sapporo in nighttime and daytime

Regions	Time of death	*n*	Pineal MT[a] (ng/PB[b])
Tokyo[c]	Night and day	41	3.56 ± 9.88[g]
	Night[e]	23	5.55 ± 12.88
	Day[f]	18	1.03 ± 1.71
Sapporo[d]	Night and day	37	7.70 ± 14.10
	Night	24	1.38 ± 16.46
	Day	13	0.94 ± 0.95

[a] MT: melatonin
[b] PB: pineal body
[c] Tokyo: N 35° 39′, E 139° 44′
[e] Night: 2000–0800 hours
[f] Day: 0800–2000 hours
[g] Mean ± S.D.

Table 7 Differences of daylight hours in Tokyo

Time of death	Month	*n*	Pineal MT[a] (ng/PB[b])
Night[c]	Aug.	3	2.11 ± 1.06[e]
	Jul. + Sep.	17	3.00 ± 3.11
Day[d]	Aug.	7	1.56 ± 2.27
	Jul. + Sep.	11	0.69 ± 0.96

[a] MT: melatonin
[b] PB: pineal body
[c] Night: 2000–0800 hours
[d] Day: 0800–2000 hours
[e] Mean ± S.D.

The samples from Tokyo were divided into 2 groups which were obtained in July and September, and in August. Furthermore, we considered the amounts of pineal MT in daytime and in nighttime, respectively. Each average is shown in Table 7, but no significant differences were recognized between MT ng/PB in August and in July and September. This result suggests that differences in daylight hours do not influence the contents of pineal MT.

The number of samples investigated here was larger than those in other reports, and was equal to the survey of Oxenkrug et al. [46].

It might be very difficult to clearly state the criteria for estimation of TOD as far as social factors are concerned, the most influencial of which is reported to be a Zeitgeber. But a recent study emphasized the light-induced interruption of MT synthesis and the effect of MT circadian rhythm in humans [47]. Therefore we cannot ignore the

influence of light, especially sunlight, and the revised times as shown in Fig.8 would have to be used.

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