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Determination of melatonin in biological samples by capillary electrophoresis

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Abstract

The determination of melatonin (MLT) in physiological samples was investigated using capillary electrophoresis (CE). Mouse blood was collected in tubes containing EDTA, centrifuged at 1500 *g* for 20 min at 4°C, and stored at −20°C. Plasma samples were extracted with dichloroethane, centrifuged and the aqueous phase was discarded. Then the organic phase was evaporated to dryness. The residue was dissolved in deionized water and filtered with a microfilter (0.22 μm). Separations were carried out using a CE system equipped with a fused silica capillary [80 cm (effective length 52 cm)×75 μm I.D.] and an ultraviolet–visible detector (200 nm), and programmed to provide 25 m*M* 2-(*N*-morpholino)ethanesulfonic acid (pH 5.7). Injection was performed hydrostatically by elevating the sample by 10 cm at the cathodic side of the capillary. The calibration curve, reproducibility, recovery and limit of detection were examined, and validation of the method was performed. The result showed that MLT in blood could be easily determined with the new method.

Keywords: Melatonin; Hormones

1. Introduction

Melatonin (MLT), *N*-acetyl-5-methoxytryptamine, is mainly secreted by the pineal gland into the circulatory system in most animal species and has several roles as a hormone [1]. The pineal gland functions as a neuroendocrine transducer, receiving light information from retina and transforming this neural signal into a chemical messenger, the best known of which is MLT [2]. This hormone is synthesized from tryptophan. Tryptophan is initially hydroxylated and then decarboxylated giving serotonin (via *N*-acetyltransferase, NAT) and a subsequent methylation (via hydroxyindole-*O*-methyltransferase) yields MLT. Circadian rhythms in pineal indole metabolism, especially for MLT, are regulated primarily by NAT [3–5]. Besides its presence in the pineal gland, MLT has been found in a variety of other tissues [6]. In recent years, many methods have been developed for the quantitation of MLT in several tissues and body fluids: bioassay [7], fluorimetry [8], gas chromatography (GC) with electron-capture detection [9], liquid chromatography (LC) with amperometric [10,11] or both, amperometric and fluorimetric detection [12], and radioimmunoassay (RIA) [13,14]. High-performance liquid chromatography (HPLC) has been used for the determination of pineal indoles because of the sim-

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plicity of sample preparation. Several authors have reported its potential use for MLT determination by means of UV–Vis [15], fluorimetric [16–19] or electrochemical detection [20,21]. However, the sensitivity and selectivity of these methods are poor because of the higher detection limit compared with other methods including our method. RIA techniques are the most widely acceptable in animal experiments. Recently, capillary electrophoresis (CE) has been introduced as a new separation technique for the determination of biological samples. The purpose of this study was to develop the best and novel analytical method of MLT in biological samples using the CE system.

2. Experimental

2.1. Materials

MLT was supplied by Sigma (St. Louis, MO, USA), dichloroethane and 2-(*N*-morpholino)-ethanesulfonic acid (MES) were purchased from Wako (Osaka, Japan). Deionized water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Equipment

A Model BioFocus 3000 CE system equipped with a UV–Vis absorbance detector set at 220 nm, a negative power supply and automatic sample changer were purchased from Bio-Rad (San Francisco, CA, USA). BioFocus system integration software (Bio-Rad) was used for data collection and treatment. Separations were carried out using a fused silica capillary [80 cm (effective length 52 cm)×75 μm I.D., purchased from Bio-Rad] at 25 kV. Injection was performed hydrostatically by elevating the sample by 10 cm at the cathodic side of the capillary. The carrier electrolyte buffer was prepared from a stock solution containing MES. This solution was diluted to variable concentrations. Some of experiments were performed using different activities of the carrier electrolyte solution. Prior to use, the pH of all carried electrolyte solutions was measured and they were then filtered through a 0.22-μm polyethylene filter.

2.3. Preparation of standard solution

A MLT stock solution was prepared by dissolving 10 mg MLT in 1 ml ethanol and 70 ml distilled water and diluting to 100 ml. The standard solution of various concentrations could be obtained by further dilution of the above prepared stock solutions. These solutions were used to obtain the calibration curve for quantitation.

2.4. Preparation of sample solution

ICR inbred male mice aged 2–3 months were maintained under a 12 h light cycle (hour 6, light on; hour 18, light off) with free access to food and water at a temperature of 23±1°C in a fully air-conditioned cabinet. Mice were divided into two groups (five to six per cage) and one group was treated orally with a daily dose of 10 mg/kg body mass of MLT at 6:00 p.m. The route of administration was oral and the treatment was continued for two weeks. Blood was collected from MLT-treated and untreated mice at night (1:00 a.m.). Mouse blood was collected in light-resistant tubes containing EDTA, centrifuged at 1500 *g* for 20 min at 4°C, and stored at −20°C. Plasma samples were extracted with dichloroethane, centrifuged at 1500 *g* for 20 min, and the aqueous phase was discarded, and then the organic phase was evaporated to dryness. The residue was dissolved with distilled water, sonicated and filtered with a micro filter (0.2 μm). The filtrate was injected into the CE system.

2.5. Relative recovery

A 100-μl volume of MLT standard solution was added to 100 μl plasma, thereafter all procedures were the same as for the sample solution.

2.6. Method validation

This new analytical method of MLT was validated with intra- and inter-laboratory (day-to-day and person-to-person) experiments.

3. Results and discussion

3.1. Optimal conditions for CE

The MLT concentration in the mouse blood is so low ranging about from 10 pg/ml to 35 pg/ml at night. The limit of detection (LOD) and the percent recovery of analytical method are critically important since LOD and recovery can be influenced by other components in the plasma, these factors have to be considered for studying on optimal analytical conditions. Electropherograms of a non-pretreated and pretreated plasma sample solutions are shown in Fig. 1, which shows that the interference of other components have been completely eliminated. The identification of the MLT peak was carried out by injection of sample solution spiked with MLT standard solution. The determination of MLT in mouse

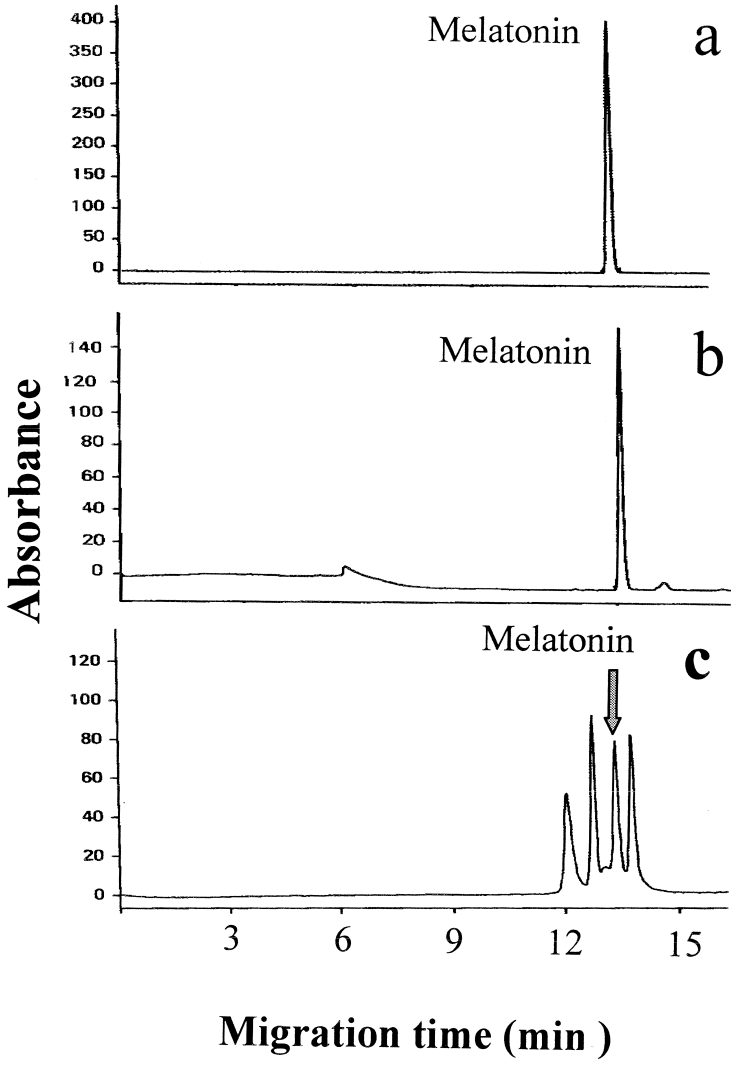

Fig. 1. Typical electropherograms of melatonin in mouse plasma samples. (a) Melatonin standard, (b) pretreated plasma sample, (c) untreated plasma sample. Analytical conditions; capillary (80 cm×50 μm), electrolyte buffer: 25 m*M* MES (pH 5.7), detector: UV (200 nm), separation strength: 25 kV, injection: 5 s at 10 kV.

plasma was performed under the optimal conditions described in Fig. 2 without any interference by other components.

3.2. Influence of voltage

The effect of varying the voltage from 5 to 30 kV was investigated using the same experimental conditions as above (Fig. 2). A potential of 25 kV yielded the best compromise in terms of run time, current generated and efficiency of separation. This potential was used in subsequent stages of method development.

3.3. Influence of electrolyte buffer molarity

The molarity of the MES buffer was varied between 10 and 50 m*M* under the same experimental

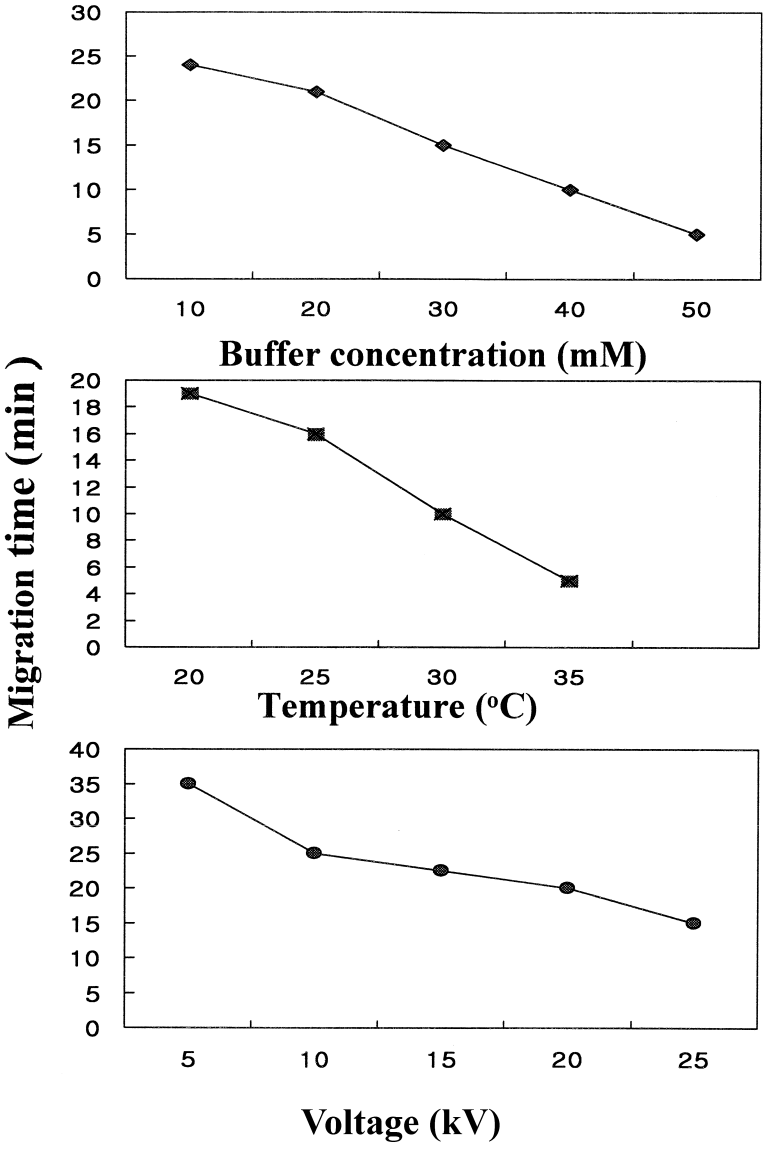

Fig. 2. The influence of analytical conditions on the migration time.

conditions as above. An increase in the buffer concentration resulted in a decrease in the electroosmotic flow (EOF) due to compression of the double layer [22] and in an increase in migration time of the solutes. Buffer concentration dramatically affected the peak shape due to a stacking effect [23]; increasing the buffer concentration up to 25 m*M* resulted in a better peak shape and efficiency, whereas higher concentrations resulted in a peak broadening due to Joule heating.

3.4. Influence of temperature

The effect of the temperature on separation was investigated between 20 and 35°C (Fig. 2).

Temperature regulation with the instrument is efficient only until 4°C below room temperature. A decrease in the temperature caused a decrease in the EOF due to lower electrolyte viscosity. The increased migration times of the solutes were due to both changes in the viscosity of the medium and partition coefficients. A temperature of 20°C was selected as it gives the best compromise between resolution and run time with an acceptable level of baseline noise.

3.5. Sensitivity and linearity

The sensitivity and linearity of the response have been also studied. The LOD by the CE technique was as low as 0.2 pg/ml MLT with a signal-to-noise ratio of 5. This LOD was about 2-fold less than that obtained with HPLC (reversed-phase column and UV detection at 220 nm) [15], and this LOD was sufficient for the determination of MLT in mouse plasma. In this study, the plasma volume needed was 10-times less than that used with HPLC method. Therefore there was no problem in the determination of mouse plasma MLT. The assay of MLT in the mouse plasma had a linear range from 5 to 50 pg/ml ($y=76.230x-1.162$, $r^2=0.9999$). MLT concentrations in mouse plasma ranged from 10 to 35 pg/ml, and mouse plasma was diluted more than 2-fold prior to analysis, linearity over the range 5–12.5 pg/ml is very important for our studies. This test was performed with a relative standard deviation (RSD) of 2.5%.

3.6. Relative recovery

The percent recovery test was conducted under the above optimum conditions. The results of the recovery test are shown in Table 1. The recovery of MLT was good, in the range 96–99%.

3.7. Influence of pretreatment

The method used for the pretreatment of plasma samples significantly affected the accuracy of the determination of MLT level. In order to study the accuracy of our technique, we tested varying extractive solvents such as dichloroethane, chloroform and diethyl ether. The data listed in Table 1 show that extractive solvents play a critical role in accura-

Table 1
The influence of plasma pretreatment on the plasma MLT level

Solvent			Sonication	Centrifugation		MLT[a] (pg/ml)
Dichloroethane	Chloroform	Methanol		*g*	Time (min)	
Yes	No	No	Yes	1500	20	32±1.5
Yes	No	No	Yes	3000	10	31±1.8
Yes	No	No	No	1500	20	30±2.1
No	Yes	No	Yes	1500	20	30±1.9
No	Yes	No	Yes	3000	10	30±2.3
No	Yes	No	No	1500	20	29±2.4
No	No	Yes	Yes	1500	20	28±2.1
No	No	Yes	Yes	3000	10	27±2.7
No	No	Yes	No	1500	20	25±2.7

[a] Each value represents the mean±standard deviation for five samples. Analytical conditions; capillary (80 cm×50 μm), electrolyte buffer: 25 m*M* MES (pH 5.7), detector: UV (200 nm), separation strength: 25 kV, injection: 5 s at 10 kV.

Table 2
Intra- and inter-laboratory validation[a]

Validation		LOD (pg/ml)	Assay (pg/ml)
Inter-laboratory	Person	3.0±1.1	26±2
	Day	3.0±1.5	26±3
Intra-laboratory	Lab. A	2.5±2.1	26±4
	Lab. B	3.0±1.5	25±3
	Lab. C	3.0±2.2	26±3

[a] Each value represents the mean±standard deviation for five samples. Analytical conditions; capillary (80 cm×50 μm), electrolyte buffer: 25 m*M* MES (pH 5.7), detector: UV (200 nm), separation strength: 25 kV, injection: 5 s at 10 kV.

cy. Each solvent extract of mouse plasma was used to determine MLT, and the effects of sonication and centrifugation were tested along with extraction. The optimum conditions for the residues were: dissolution in dichloroethane and sonication, and thereafter centrifugation at 1500 *g* for 20 min.

3.8. Determination of MLT in mouse plasma after oral administration

There were differences between post absorptive plasma MLT level and that before administration. That is, plasma MLT level increased by 2–3 pg/ml. This result suggests that this analytical method can be used to determine MLT in the physiological samples for pharmacological and toxicological studies on MLT.

3.9. Method validation

The intra- and inter-laboratory validation was conducted with the newly developed analytical method of MLT. Table 2 shows the relatively good results of these validations. Intra-laboratory validation was not as good as inter-laboratory validation, because of the difference of equipment (one laboratory: Model HP ^{3D}CE system, Hewlett-Packard, CA, USA, another laboratory: Model Waters Quanta 4000 CE system, Waters, CA, USA).

4. Conclusions

A CE technique for the quantitative determination of plasma MLT has been developed. The method was sensitive, rapid, accurate and only 100 μ1 plasma sample was required. The separation took only 20 min and the components in blood did not interfere with separation of MLT. The LOD of MLT was 1 pg/ml. Plasma pretreatment procedure was simpler than those of HPLC technique and conventional spectrophotometric method. Extraction of plasma sample with dichloroethane was most sensitive and accurate compared with other organic solvents. Relative recoveries of MLT ranged between 96–99%. We suggest that this method could be applied for the determination of MLT in plasma.

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