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**—Research Note—**

# Development and Evaluation of a Rapid Enzyme-immunoassay System for Measurement of the Urinary Concentration of Estrone-3-glucuronide in a Female Giant Panda (*Ailuropoda melanoleuca*)

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**Abstract.** To detect estrus for reproductive management, and to determine the relationship between urinary estrogen and estrous behavior, in a female giant panda, we developed and evaluated a rapid enzyme immunoassay (EIA) system for urinary Estrone-3-glucuronide (E1G) using commercial reagents. The developed EIA system took only around 3 hours, including all procedures to obtain a result. It indicated good reproducibility (intra-assay CV of 5.16%, interassay CV of 15.4%) and sensitivity (lowest standard concentration was 0.0156 ng/ml) for measurement of the urinary concentrations of E1G in the giant panda. There was a positive correlation (r=0.934) with the data for estrone (E1) in the same samples, as measured by radioimmunoassay (RIA) performed in a commercial laboratory. The changes in the E1G concentrations were almost synchronous with the changes in E1 assayed by RIA in urine collected during 4 consecutive estrous seasons. The dynamics of urinary E1G measured by this system highly correlated with the occurrence of the presenting estrous behavior in the giant panda. The above results indicate that this assay system may be normally, rapidly and practically used for measurement of the urinary concentration of E1G in the giant panda.
**Key words:** Giant panda (*Ailuropoda melanoleuca*), Enzyme immunoassay, Estrone-3-glucuronide, Estrous behavior

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The giant panda (*Ailuropoda melanoleuca*) is a critically endangered species. Its natural habitat is located and limited to Western China at elevations of 2,000 m to 4,000 m. The actual population is estimated to be no more than 1,500 individuals [1]. The giant panda is categorized as "Endangered" on the Red List of Threatened Species published by the International Union for Conservation of Nature and Natural Resources [2].

Female giant pandas have a single annual estrus period and generally are in estrus for only a few days between February and May [3]. This characteristic constitutes a major limitation on production of new born in giant pandas.

At the Kobe Municipal Oji Zoo, a collaborative project concerning reproduction of the giant panda has been conducted with China since 2000. Since the main purpose of this project is production of baby pandas, the primary work required is establishment of a rapid assay system to measure the sex steroid hormones to evaluate the giant panda's reproductive status and especially to detect estrus by measuring estrogen concentrations in the female for natural and artificial breeding.

Because zoo and wild animals are not domesticated or are untrained, frequent sampling of blood is very difficult, and so urine or feces, which are easily collected without being invasive to the animals, are used to measure hormones. In Japan, another study of reproduction of the giant panda has been underway at Ueno Zoological Gardens, Tokyo, since 1972. They have succeeded in production of giant panda by artificial insemination (AI) [4]; however, they have also experienced unsuccessful reproduction in a female giant panda despite efforts to utilize AI for 10 years [5]. In these studies, they have also used urine to measure estrogen. Furthermore they have used radioimmunoassay (RIA) to measure urinary hormones in their studies. RIA is traditional and reliable method of measuring hormones, however, because we need special limited space where isotope is available to perform RIA, RIA is not really for measurement hormones in practical institutes, such as zoos.

In recent years, enzyme immunoassay (EIA) has become a common method for measuring steroid hormones in urine and feces of the domestic [6–10], zoo and wild animals [9, 11–17]. One of the pioneer studies on development of non-invasive EIA in zoo animals is the work of Czekala *et al*. [13], who assayed the urinary estrone conjugates of several zoo animals. This original EIA method has been used to assay progesterone concentrations in the plasma of the sheep, cow, horse, goat, dog and cat by Munro and Stabenfeld [18]. MacGeehan developed an assay system for urinary estrone-3-glucuronide (E1G) and pregnanediol-3-glucuronide in the giant panda by modifying the methods of Munro *et al*. [19] and French *et al*. [20]. However, it is difficult for researchers working in different institutions to use the same EIA systems due to the difficulty of obtaining the specific antisera used in the systems. Therefore, in the present study, an easy and rapid EIA was established for measuring urinary E1G in a female giant panda using commercial reagents to evaluate her reproductive status.

This study aimed to establish an easy and rapid EIA system for

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measuring urinary E1G in a captive female giant panda. The relationship between urinary concentrations of E1G and the intensity and length of presenting behavior during the breeding seasons in 6 consecutive years were also evaluated.

## Materials and Methods

*Animals*

A female giant panda named "ShuangShuang", born at Wolong Natural Reserve in China on September 16, 1995, was loaned by China to Kobe Municipal Oji Zoo (Kobe, Japan) on July 16, 2000. A partner was kept in a neighboring enclosure throughout the study period, July 2000 to April 2006. The partner kept from July 16, 2000 to December 5, 2002 was female. Between December 2002 and April 2006, the female partner was replaced with a mature male named Long-Long, who was born at Wolong Nature Reserve on September 14, 1995 and on loan to the zoo since December 9, 2002.

*Management of animal*

ShuangShuang was kept in as enclosure consisting of an indoor space (128.5 $m^2$), including exhibition, bed and nesting rooms, and an outdoor space (283.5 $m^2$). The neighboring space was reserved for her partner from which she was separated by stainless slits except when they were mixed for natural mating. From 0730 h to 1500 h, she could alternatively utilize both the indoor and outdoor spaces and could only utilize the indoor space from 1500 h to 0730 h. She was fed bamboo, carrots, apples and pellets (Mazuri 5MA4, PMI Nutrition International, East Logan, UT, USA) 6 times a day with permanent access to drinking water in an indoor or outdoor pool.

*Collection of urine samples*

Daily urine samples was collected from ShuangShuang as a pooled sample flowing into a collection cup that was set beneath the drainage hole of the indoor bed room from 1700 h to 0900 h of the next day during each estrus season (March–May) between 2001 and 2006. If the collecting cup was empty at 0900 h, the collecting cup was left in place during the daytime until urination. When the urine in the cup was suspected of being contaminated with water, urine samples were immediately collected after urination by aspiration with a plastic syringe from the floor of the indoor space during the daytime. Collected urine samples were centrifuged for 4 min at 650 × g, and the supernatant was stored at –40 C until assay.

*Hormone assays*

Urinary concentrations of E1G and E1 were measured by EIA and RIA, respectively. The E1G assay was carried out using a double antibody enzyme immunoassay. The 1st antibody, 2nd antibody, enzyme linked antigen and standard used in the present study were as follows, respectively: anti-estrone-3-glucuronide-BSA (No. FKA224-E; Cosmo Bio, Tokyo, Japan), anti-rabbit IgG (H + L) (No. 270335; Seikagaku, Tokyo, Japan), estrone-3-CME-HRP (FKA223, Cosmo Bio) and estrone-3-($\beta$-D-glucuronide) (E1752, Sigma-Aldrich, St. Louis, MO, USA). The cross-reactivity of the 1st antibody was as follows: 100% for estrone; 170% for E1G; 25% for estrone-3-sulfate; 1% for estradiol; 0.1% for estriol; 1.2% for estradiol-3-glucuronide; 0.1% for estradiol-3-sulfate; 0.05% for testosterone; 0.07% for 4-androstenediol; 0% for progesterone; 0 % for cortisol and 0% for 17-$\alpha$-OH-progesterone. The 2nd antibody (100 $\mu$l of 26.47 $\mu$g/ml) was diluted with coating buffer (2.574 g $Na_2CO_3$-$10H_2O$, 1.758 g $NaHCO_3$ in 600 ml ultra pure water, pH 9.6) and added to a 96-well microplate. The plates were incubated at room temperature for 2 h, and then the wells were completely emptied. EIA buffer (250 $\mu$l, 7.12 g $Na_2HPO_4$-$2H_2O$, 8.5 g NaCl in 1,000 ml ultra pure water, pH 7.2) was added to each well. The plates were then incubated at room temperature. All plates were sealed and stored in a refrigerator until use. The plate coated with 2nd antibody was washed with wash buffer (1 g Tween 80 in 1,000 ml distilled water) 2 times. Then, 20 $\mu$l of standards (0.015625–2 ng/ml), diluted samples with EIA buffer and control urinary sample (dilution rate: × 100, collected from ShuangShuang on April 30, 2003) were added to each well in duplicate. On the same plate, 120 $\mu$l and 20 $\mu$l of EIA buffer were added to non-specific binding wells and maximum binding wells in duplicate, respectively, and two wells were prepared as Blank wells. Next 100 $\mu$l of the 1st antibody (final working dilution: × 50,000,000) was added to each well except for non-specific binding and blank wells, and 100 $\mu$l of HRP binding antigen (final working dilution: × 500,000) was added to each well, except for the well. After adding all of the standards, samples, the control sample and reagents, the plate was incubated with shaking at 150 rpm in 20 C for 2 h. After washing with wash buffer 4 times, 150 $\mu$l of substrate buffer, which consisted of solutions A (0.25 g urea hydrogen peroxide, 4.5 g $Na_2HPO_4$-$2H_2O$, 2.575 g citric acid-$H_2O$ in 250 ml ultra pure water) and B (0.125 g tetramethylbentidine, 10 ml dimethylsulfoxide, 2.575 g citric acid-$H_2O$ in 240 ml ultra pure water) were mixed at a ratio of 1:1, and the combined solution was added to each well. The plate was then incubated in a shaking dark incubator at 37 C for 40 min. Fifty microliters of 4 N-$H_2SO_4$ was then added to each well to stop the reaction before reading the plate at a wave length of 450 nm with a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA). The samples were measured using a plate for each year between 2001 and 2006, and so a total of 6 assays were performed. Each urine sample was diluted 10-fold (before and after estrus) or 100-fold (during presenting estrous behavior) with EIA buffer before assay. Computer software (Microplate Manager 5.0 PC, Bio-Rad Laboratories) was used to calculate the E1G concentrations of standards, each sample and the control sample. The standard curve was fitted to a cubic curve adopted in the software. The intra-assay coefficient of variation (intra-assay CV) was calculated from triplicate assay using the control sample. The interassay coefficient of variation (interassay CV) among the 6 assays was calculated from the data for the control sample. To evaluate the effect of urinary components on the sensitivity and reproducibility of the assay, known concentrations of E1G (0.015625–2 ng/ml) were arranged with EIA buffer only, or EIA buffer containing urine diluted 10- and 100-fold, respectively. The urine added to the standards was collected from ShunagShuang on June 13, 2003 in early period of pseudopregnancy 44 days after the E1G peak in 2003. To evaluate the parallelism between the dose response curve of serial dilutions of the giant panda's urine and the standard curve, urine

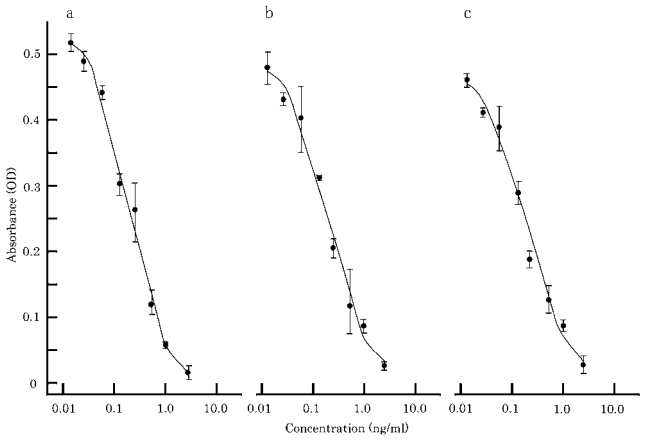


**Fig. 1.** Competitive curves of E1G in EIA buffer with or without diluted urine. E1G standards (0.015625 to 2 ng/ml) were dissolved in EIA buffer only (a), EIA buffer containing urine diluted 100-fold (b), and EIA buffer containing urine diluted 10-fold (c). Each curve was expressed as according to the following formulas. a: OD= – 0.07243 + 1.137 C – 0.6459 $C^2$ + 0.09372 $C^3$. b: OD = 0.007771 + 0.9263 C – 0.5425 $C^2$ + 0.08028 $C^3$. c: OD = 0.1076 + 0.7273 C – 0.4433 $C^2$ + 0.065556 $C^3$. OD: Optical Density at 450 nm, C: Log (Concentration of standard).

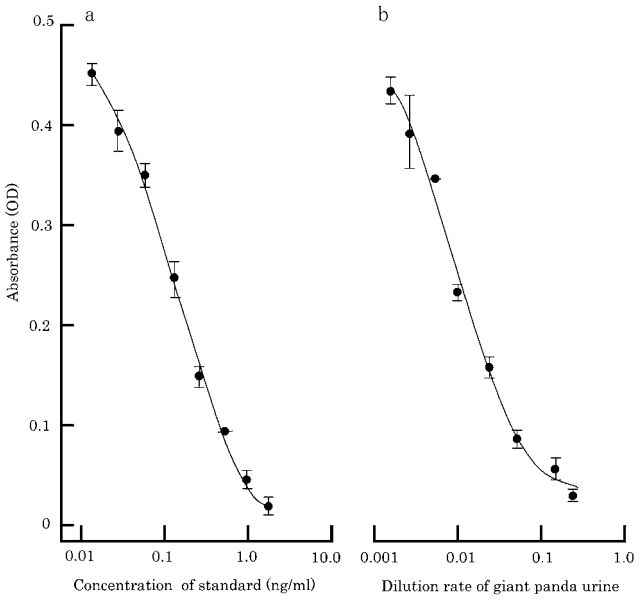


**Fig. 2.** Dose response curve of a serial diluted urine sample (b) compared with a standard curve (a). a: OD = 0.1629 + 0.6841 C – 0.4577 $C^2$ + 0.07193 $C^3$. b: OD = 0.1490 + 0.3548 C + 0.3087 $C^2$ + 0.05262 $C^3$. OD: Optical Density at 450 nm, C: Log (Concentration of standard in a and diluted sample in b).

collected on March 20, 2004, the day that the concentration of E1G reached to the peak value during the estrous period in 2004, was used to measure the E1G concentrations of serial dilutions (×4–×800). E1 assays were performed using a commercial RIA Kit (Estrone RIA DSL-8700; Diagnostic Systems Laboratories, Webster, TX, USA) at a commercial laboratory (Mitsubishi Kagaku Bio-Clinical Laboratories, Hyogo, Japan) from 2001 to 2004. The urinary creatinine concentration was measured using a modified-Jaffe's reaction as follows. Each urine sample, control sample and creatinine standard (the creatinine powder dissolved with 0.1N-HCl to a concentration of 1 mg/ml) was diluted 101-fold with distilled water. Standard, blank, samples and the control sample were assayed with 4-replicates in a 96-well microplate. One hundred microliters of distilled water was added to the blank well, 100 $\mu$l of diluted sample was added to each sample well, 100 $\mu$l of diluted control sample was added to each control sample well and 100 $\mu$l of diluted standard was added to each standard well. Fifty microliters of 0.75 N NaOH and 50 $\mu$l of 0.04 N-picric acid were added to each well, respectively. The plate was then placed in an activated microplate reader for 15 min before reading at wavelengths of 490 and 630 nm. The creatinine concentrations (mg/ml) of samples and the control sample were calculated by comparison between the absorbance data of the samples/control sample and that of standard. Finally, the concentrations of E1G and E1 were calculated as ng/mg of creatinine (Crmg).

*Observation of presenting behavior*

Presenting behavior, defined as tail-up to expose the vulva to a male mating partner or others, i.e., the keepers, was observed as an indicator of estrus behavior in ShuangShuang. This behavior was continuously observed 24 h daily by video camera throughout the experiment and is indicated as the number of presenting behaviors in a 24 h period.

*Statistical analysis*

The concentrations (ng/Crmg) of E1G and E1 of each sample were analyzed with SPSS Ver.10 (SPSS, Chicago, IL, USA) to compare the correlation coefficients between E1G and E1 (n=63).

## Results

The standard curves for EIA buffer only and EIA buffer containing the panda's urine diluted 10- and 100-fold are shown in Fig.1. The absorbance of each concentration of standard slightly decreased as the concentrations of additional urine increased. The concentration of E1G of the urine that was added to the standard was 0.028 ng/ml at 10-fold dilution and was undetectable at 100-fold dilution in this assay system. The fitness to the curve for both sets of standards containing urine (10- and 100-fold) was good. The intra-assay CV was 5.16%, and the interassay CV among the 6 assays was 15.4%. The dose response curve for urine containing high concentrations of E1G paralleled the standard curve (Fig. 2).

As the data for the concentrations (ng/Crmg) of E1G and E1 were found to not be normally distributed (Kolmogorov-Smirnov's Z=0.232 for E1G and 0.212 for E1, P<0.05), the Spearman's rank correlation coefficient was calculated to be 0.934. Moreover, as these data were found to be normally distributed (Kolmogorov-Smirnov's Z=0.063 for E1G and 0.097 for E1, P<0.05) as a result of log-transformation, the Pearson's correlation coefficient was calculated to be 0.904.

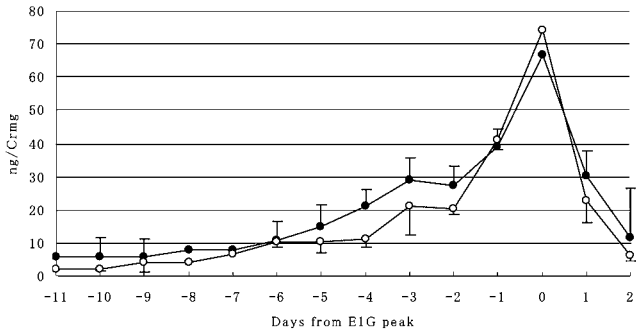


**Fig. 3.** Changes in the mean urinary concentrations of E1G (●) and E1 (○) around the estrous period between 2001 and 2004. Each bar indicates the standard error.

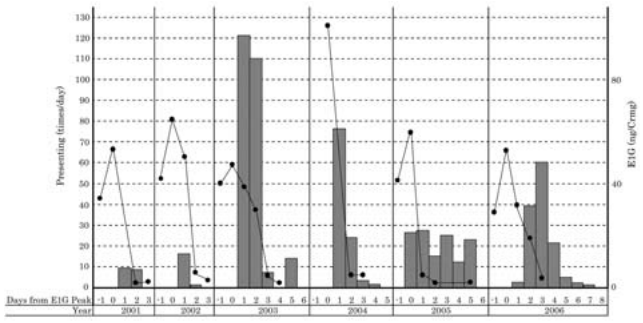


**Fig. 4.** Relationship between urinary E1G concentrations (ng/Crmg) and frequency of presenting behavior for each year. ———: Urinary E1G concentration. ▬: Presenting behavior.

The changes in the mean concentrations of E1G and E1 from 2001 to 2004 are shown in Fig 3. The changes of E1G matched well with those of E1. The mean ± SD of the peak values in E1G was 63.0 ± 19.9 ng/Crmg. The peak concentrations of urinary E1G fluctuated from 47.4 ng/Crmg to 101.7 ng/Crmg over the course of 6 years.

The presenting behavior started on the day the urinary E1G concentrations decreased after the peak (=day 1) in each year except for 2005 when it started on the day that the E1G concentration reached the peak (day 0; Fig.4). The frequencies of presenting were low in 2001 and 2002 when the neighboring partner was female, while the presenting behavior was observed more frequently between 2003 and 2006 when a mature male was present. From 2001 to 2004, the presenting behavior tended to occur on day 1 and day 2, while in 2005 and 2006, it lasted for more than 3 days. The frequency and length of the presenting behavior were not correlated with the peak values of the urinary E1G concentrations.

## Discussion

Endocrinological studies have revealed that the concentration of estrogen conjugate (EC) in plasma and urine increases during the follicular phase and then decreases a few days before ovulation in giant pandas [21] and mares [8]. In mares, the concentration of EC in the urine is 10-fold higher than that in the plasma due to the kidney concentrating capacity and the resistance of EC metabolites to rapid degradation [22]. Since estrogen is not stored in the body, EC excreted in urine has been shown to be an index of estrogen secretion [23]. E1G is one of the principal EC components in urine [24]. Thus, urine samples seem to represent an adequate material for estrogen assays that can help in estimating the estrus and ovulation times of wild and domestic animals.

In the present study, addition of urine containing low concentrations of E1G to the standards resulted in a slight decrease in the optical density (absorbance) compared with those of standards diluted with assay buffer only. Since the urine samples added to standards contained very low concentrations of E1G, the decrease in absorbance may be due to the presence of some factors in the urine that inhibit the reaction between the first antibody and E1G and/or between first antibody and second antibody. However, the standard curves containing diluted urine were well-fitted to the cubic curve as well as the standard curve without the addition of urine, and therefore inhibitors did not affect the quality of the assay. This was also confirmed by the results for the dose response curve, which showed that the serial diluted samples paralleled the standard curve.

The reproducibility of the present EIA system was evaluated as good based on intra- and interassay CVs of 5.16 and 15.4%, respectively. Another experiment was conducted to compare the urinary concentration of E1G obtained in our system to that of E1 using a commercial RIA kit in a commercial laboratory. The concentration of E1 by EIA was positively correlated with the concentration of E1 measured by RIA. In addition, the changes were synchronized. The above results show that the EIA system is practical for measuring urinary E1G concentrations.

In this study, the peak concentration of E1G in each estrous season was not correlated with the frequency or duration of presenting behavior. At the beginning of this study (2001–2002), Shuang-Shuang showed low frequency of presenting behavior for a few days after the peak of E1G. This seems to be due to the fact that the partner was female. Interestingly, the length and frequency of presenting behavior markedly increased when a mature male was introduced in 2003. Since the peak concentrations of urinary E1G were not associated with the frequency and duration of presenting behavior during the 6 consecutive estrous seasons, the estrous behavior in the female giant panda appeared to be influenced not only by the estrogen concentrations but also by environmental circumstances, including the presence of a sexually mature male. This result may be somehow comparable to the ram effect reported by Rosa *et al.* [25]; basically, the presence of a sexually mature ram is the key to inducing estrus and ovulation in anestrous ewes.

In giant pandas, “tail up” behavior, defined as presenting behavior in our study, was observed from Day -1 until Day 5 (Day 0=day on which the urinary concentration of E1G reached a peak) [21]. Murata *et al.* [26] demonstrated that the female presents estrous behavior in the presence of a male 1 day after the urinary estrogen concentration reaches a peak. In the present study, the correlation between E1G and the presenting behavior was in agreement with the results of the above reports [21, 26]. The fact that our data on the E1G concentrations and presenting behavior matched those of

previous reports [18, 23] proves that our EIA system is suitable for measuring urinary E1G to detect estrus in the female giant panda.

Prediction of estrus and ovulation based on these types of changes in the E1G concentration has been reported in several previous studies [27–30]; however, the previous methods used for measuring steroids, such as RIA, involved time-consuming, risky and expensive procedures [22, 27, 31]. The EIA system developed in the present study, however, is a simple and fast method (3 h) for measuring daily urinary E1G. Since the dynamics of urinary E1G measured by this EIA system was highly correlated with the occurrence of the presenting estrous behavior, this system may be routinely used to measure the urinary concentrations of E1G in the giant panda.

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