高速液体クロマトグラフィーによる犬の血清グルココルチコイドの測定および正常犬におけるコルチゾール値の日内変動パターン

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Measurement of Serum Glucocorticoids by High-Performance Liquid Chromatography and Circadian Rhythm Patterns of the Cortisol Value in Normal Dogs

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The concentration of cortisol in canine peripheral blood has usually been measured by various methods, such as fluorometric assays [3, 6], competitive assays [5, 6], and radioimmunoassays [2, 4]. Of these, the radioimmunoassay is thought to be most specific method and is used frequently for the determination of steroids. However, its specificity seems to be still doubtful because some cross reactions with other steroids, such as 11-deoxycortisol, corticosterone, and cortisone, may occur [2]. Recently, high-performance liquid chromatography (HPLC) has been employed for the determination of serum steroids [9, 12]. The HPLC can precisely detect each steroid in the serum simultaneously and promptly, without any interactions with other steroids. Thus, the HPLC method of estimating the steroids has the advantages of great specificity and simplicity over other methods. However, to obtain the optimum extract for the HPLC analysis, adequate pre-treatments of the sample have been required; for example, repeated shaking of the sample with an organic solvent is needed to separate the steroids from water-soluble compounds, which is time-consuming and laborious. Furthermore, in these processes, the formation of emulsions in the sample occurred, which often results in a considerable loss of each steroid in the sample. In the present study, we employ a column of granular Kieselgur (Extrelut®1, Merck) [9] for separating the steroids from serum.

Fourteen clinically healthy dogs of mixed breeds, 6 months to 9 years of age (6 females and 8 males) were used. Each dog has been raised individual cage at least for 2 years, except 3 dogs which were born in this laboratory 6 months ago. They were maintained in a regular dog diet at noon and water *ad libitum*. Blood (1.5 m*l*) was drawn from the cephalic vein at 6-hour intervals for one day (3:00, 9:00, 15:00, and 21:00). The serum was separated immediately after each blood collection and stored at −20°C until use. A 0.5 m*l* aliquot of serum was mixed with 0.5 m*l* of 50 mM sodium phosphate, pH 8.0, containing 20 ng of 19-nortestosterone (Sigma) as an internal standard [9]. The mixture was applied to the Extrelut® 1, and ten minutes later, the column was eluted with 6 m*l* of dichloromethane. The eluate was evaporated and the residue was dissolved in 0.5 m*l* methanol, and evaporated again to dryness. The resulting residue was dissolved in 200 μl of the mobile phase of the HPLC, and filtered through a Millipore SJHV membrane (pore size, 0.45 μm), after which 100 μl was through an HPLC system from Waters Associates. Isocratic elution with methanol/acetonitrile/water (55:3:42, v/v), was carried out at a flow rate of 1.5 ml/min. The eluate was monitored for ultraviolet absorption at 246 nm and the concentrations of steroids were quantified using commercially obtained steroids (cortisol, cortisone, and corticosterone) as standards. The 19-nortestosterone was used as an internal standard.

Fig. 1 shows a chromatogram of a dog serum sample. In all samples, cortisol was clearly detected and was the predominant serum glucocorticoid. The serum cortisol values of 14 dogs varied from 0.17 to 3.69 $\mu g/dl$ with a mean and standard deviation value of 1.10 ± 0.74 $\mu g/dl$. The mean cortisol value of normal dogs in this study was similar to those reported by previous workers using radioimmunoassays [2, 4], but lower than those using competitive protein binding assays [5, 6], or fluorometric assays [3, 6]. The cortisone was also detected in 6 dogs with a maxmum value of 0.57 $\mu g/dl$. Corticosterone was not quantified by the present analytical procedure. There were no significant correlations between the cortisol values obtained and the sex and age of dogs.

As compared to conventional extraction methods described elswhere, Extrelut®1 permits the separation of the steroids in a single step with

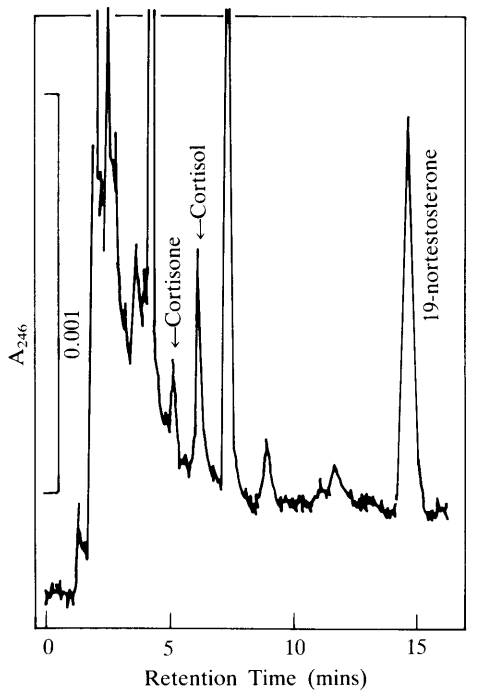

Fig. 1. A chromatogram of serum glucocorticoids in a dog.

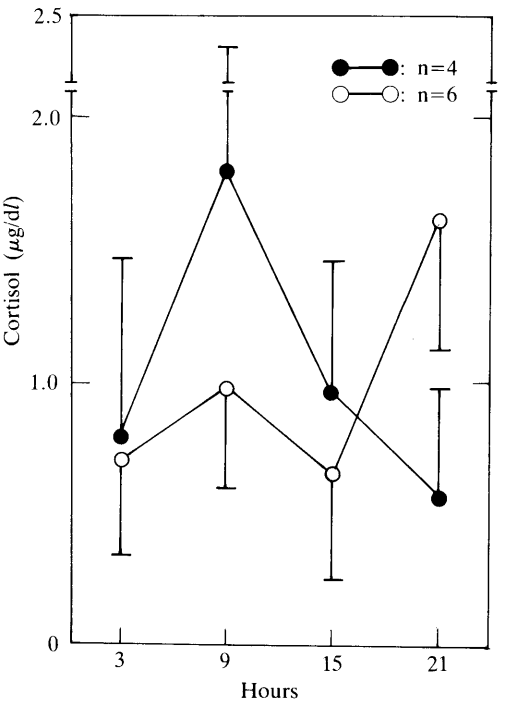

Fig. 2. Two circadian patterns of the serum cortisol values in normal dogs.

smaller expenditure of time and work [1, 9, 11]. Formation of emulsions in samples, which often obscure the estimation of steroids using HPLC are eliminated. Thus, it is thought that cortisol values obtained in the present study are more precisely quantified than those reported previously.

As shown in Fig. 2, two different circadian patterns of the serum cortisol values were demonstrated in 10 dogs, while the remaining 4 dogs showed no clear circadian pattern of the cortisol value. That is, four dogs showed a high cortisol value in the morning and a low value in the evening. In contrast, 6 dogs showed an inverse pattern of the fluctuations of cortisol values. There was no correlation between cortisol values and cortisone values.

In recumbent normal man, the cortisol level has been reported to exhibit a circadian rhythm showing a high value towards the end of sleep and a low value in the evening [8]. In rats, it was demonstrated that the circadian rhythm of corticosterone (the predominant glucocorticoid in rats) values showed a low value during the early morning and a high value just prior to the dark phase [7].

In dogs, Rijnberk *et al.* [10] found a circadian rhythm for plasma 11-hydroxycorticosteroid values, estimated by the fluorometric method. There was a tendency to show a lower value in the morning than in the evening in eleven housetrained dogs, and a higher value in the morning in sixteen laboratory dogs. However, they had no explanation for this difference. Campbell *et al.* [3] reported that cortisol values in normal dogs estimated by the fluorometric method showed a circadian rhythm with a high value at 10:00 and a low value at 22:00, and that the difference between the two values was statistically significant. Furthermore, they observed that several dogs with adrenal hyperactivity showed a reversal of the normal circadian rhythm. In the present study, two circadian patterns of serum cortisol values were found in clinically healthy dogs kept under the same conditions. The cause of this phenomenon is unclear at present.

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要　　約

高速液体クロマトグラフィーによる犬の血清グルココルチコイドの測定および正常犬におけるコルチゾール値の日内変動パターン（短報）：村瀬敏之・稲葉　睦・前出吉光（北海道大学獣医学部家畜内科学教室）——血清の前処理に脂溶性画分抽出用カラム (Extrelut®1, Merck) を用いて簡便化し，検出系にHPLCを用いることで特異的にグルココルチコイドの測定を行うことができた．14頭の正常犬で血清コルチゾール値は1.10±0.74 μg/d*l* (平均± S.D.)，また，うち6頭でコルチゾンが検出されその最大値は0.57 μg/d*l* であった．さらに，コルチゾール値の日内変動パターンを異にする二つの個体群が観察された．