



Sensitive radioimmunoassay of total thyroxine (T4) in horses using a simple extraction method

Siriwan TANGYUENYONG^{1,2)}, Yasuo NAMBO^{1,3)}, Kentaro NAGAOKA^{1,2)},
Tomomi TANAKA^{1,4)} and Gen WATANABE^{1,2)*}

¹⁾Department of Basic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-1193, Japan

²⁾Laboratory of Veterinary Physiology, Cooperative Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

³⁾Department of Clinical Veterinary Science, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido 080-0834, Japan

⁴⁾Laboratory of Veterinary Reproduction, Cooperative Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

ABSTRACT. Most thyroid hormone determinations in animals are based on immunoassays adapted from those used to test human samples, which may not reflect the actual values of thyroid hormone in horses because of the presence of binding proteins. The aims of the present study were i) to establish a novel radioimmunoassay (RIA) using a more simple and convenient method to separate binding proteins for the measurement of total thyroxine (T4) in horses and ii) to validate the assay by comparing total T4 concentrations in yearling horses raised in different climates. Blood samples were collected from trained yearlings in Hokkaido (temperate climate) and Miyazaki (subtropical climate) in Japan and from adult horses in estrus and diestrus. T4 was extracted from both serum and plasma using modified acid ethanol cryo-precipitation and sodium acetate ethanol methods. Circulating total T4 concentrations were determined by RIA. T4 concentration by sodium acetate ethanol was appropriately detectable rather than sodium salicylate method and was the same as for acid ethanol method. Furthermore, this sodium acetate ethanol method required fewer extraction steps than the other methods. Circulating T4 concentrations in yearlings were 225.98 ± 20.89 ng/ml, which was higher than the previous reference values. With respect to climate, T4 levels in Hokkaido yearlings tended to be higher than those in Miyazaki yearlings throughout the study period. These results indicated that this RIA protocol using a modified sodium acetate ethanol separation technique might be an appropriate tool for specific measurement of total T4 in horses.

KEY WORDS: climatic differences, horse, radioimmunoassay, total thyroxine, yearlings

J. Vet. Med. Sci.

79(7): 1294–1300, 2017

doi: 10.1292/jvms.17-0133

Received: 15 March 2017

Accepted: 24 May 2017

Published online in J-STAGE:
11 June 2017

The thyroid gland synthesizes and secretes the thyroid hormones 3, 5, 3', 5'-tetraiodothyronine (or thyroxine; T4) and 3, 5, 3'-triiodothyronine (T3), which play important roles in thermogenesis, growth and metabolism in animals [1, 2, 4, 34]. T4 is transformed to T3, the active form of the hormone, by monodeiodination in peripheral tissues [27]. Although over 99% of thyroid hormones circulating in blood are bound to plasma proteins, only the free (f) forms, fT3 and fT4, bind to thyroid hormone receptors [5, 12, 21, 25].

Several techniques are routinely used to evaluate thyroid hormone concentrations in various samples (*e.g.*, plasma, serum, saliva and feces) for research and the diagnosis of thyroidal or non-thyroidal disease in humans and animals. Radioimmunoassay (RIA) is considered the gold standard method to determine thyroid hormone levels, because RIA exhibits high sensitivity and specificity and low detection limits [2, 14, 15, 20, 21, 29, 30]. Furthermore, equilibrium dialysis [2], ultrafiltration [31], enzyme immunoassay [3, 23, 33, 34], chemiluminescence immunoassay [10, 28], chemiluminescent enzyme immunoassay [11] and electrochemiluminescence immunoassay [10, 39] are available to evaluate thyroid function in many species. Nonetheless, reference values of thyroid hormone concentrations vary substantially by measurement techniques used and laboratories performing the assays.

*Correspondence to: Watanabe, G., Laboratory of Veterinary Physiology, Cooperative Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan. e-mail: gen@cc.tuat.ac.jp, watagen04@ezweb.ne.jp

©2017 The Japanese Society of Veterinary Science



This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)

Recently, various commercial immunoassay kits developed for human samples have been widely applied to determine T4 and T3 concentrations in animals, including horses, for biomedical research and clinical hospital practice, because of the simplicity of measurement procedure. Thyroid hormones are chemically similar in humans and horses. However, thyroid hormone binding proteins, such as thyroid hormone-binding globulin, albumin and thyroxine-binding prealbumin (TBPA), differ between species in their quantities and binding affinities [9]. Although circulating concentrations of total T4 reflect T4 bound to binding proteins and a small amount of free T4 (about 0.05% of total T4). Importantly, however, a determination of serum fT4 levels in horses does not provide any information on thyroid gland function that is additional to that of total T4 measurement [30]. In addition, differences in binding proteins between species can affect the accuracy of T4 detection by kits that have been produced specifically for humans. Therefore, an assay optimized to measure total T4 in horse that includes the dissociation of T4 from binding proteins is necessary to determine the true concentration of circulating T4 in horses.

The aims of this study were: i) to establish a novel and reasonable RIA protocol using a more simple and convenient technique to remove binding proteins for equine T4 measurements and ii) to evaluate the assay by comparing total T4 concentrations in yearling horses raised in different climates.

MATERIALS AND METHODS

Animals

All procedures in this study were conducted in accordance with the guidelines of the Institutional Animal Welfare and Experiment Management Committee of the Japan Racing Association (JRA) Hidaka Training and Research Center.

Trained yearling and adult thoroughbred horses in estrus or diestrus were used in this study. The yearlings were housed in Hokkaido (in the temperate north) and Miyazaki (in the subtropical south), Japan, under natural light conditions from December to April (mid-December, late January, late February, mid-March and early April). Blood samples were collected from jugular veins into non-anticoagulant and heparinized vacutainers. After centrifugation, sera and plasma were harvested and stored at -20°C until assayed. To investigate T4 levels in different methods of binding protein separations, we obtained plasma samples from Hokkaido yearlings during the winter season (December). Sera collected from adult horses in their estrous and diestrous periods were used for testing using the sodium salicylate method. Plasma from Hokkaido and sera from Miyazaki yearlings under natural light conditions were also employed for different climate comparisons.

Separation of binding proteins

Sodium salicylate method: The use of sodium salicylate for binding protein separation in an evaluation of thyroxine has been described previously [35]. A glycine-gelatin buffer (GGB) containing 2% sodium salicylate (w/v, Wako Pure Chemical Industries, Ltd., Osaka, Japan) has typically been used as a T4 assay buffer to dilute the standard, samples, antibodies and T4 radioligand. In the present study, this original GGB was used to prepare standard and sample solutions for the dissociation of T4 from proteins in the assay buffer and samples. Then, GGB which was modified from Tohei *et al.* [35] by adjusting gelatin to 0.1% (w/v); with sodium azide 0.1% (w/v, Sigma-Aldrich Co. LLC., St. Louis, MO, U.S.A.) added, pH titrated to 7.4, and without sodium salicylate, was required to mix with standard and sample solutions in the RI assay. In addition, to investigate the effect of sodium salicylate on the binding of T4 radioligand to primary antibody, the original GGB was serially diluted with modified GGB to make dilutions containing 1 to 0.002% sodium salicylate, which were assayed as RIA samples.

Acid ethanol cryo-precipitation: The following extraction method was modified from a method commonly used before IGF-1 measurement [6]. Standards and samples (100 μl) with acid ethanol mixture (400 μl), which was prepared with 2 M HCl and 99.5% ethanol at a ratio of 12.5:87.5 (v/v), were mixed in $12 \times 75\text{-mm}$ glass tubes and incubated at room temperature for 30 min. After centrifugation at $2,100 \times g$ and 4°C for 30 min, supernatants were collected by decantation and neutralized with 0.855 M Tris (hydroxymethyl) aminomethane (Sigma-Aldrich Co.) solution at a ratio of 5:2, mixed thoroughly and then stored at -20°C for 1 hr. After storage, all tubes were immediately centrifuged at $2,100 \times g$ and 4°C for 30 min. The supernatants were collected into fresh tubes and diluted with gelatin-phosphate buffered saline (PBS), which was composed of 0.05 M PBS containing 0.1% sodium azide, 0.1% gelatin and 0.1% Triton X-100 (polyoxyethylene octylphenylether) (Sigma-Aldrich Co.), pH 7.4, to obtain the final solutions; and then stored at -20°C until assayed.

Sodium acetate ethanol method: This technique using alcohol and sodium acetate is commonly used for protein precipitation [26]. The standard and samples (100 μl) were transferred to glass tubes. A sodium acetate ethanol mixture (300 μl) of 2 M sodium acetate (Wako Pure Chemical Industries, Ltd.) and 99.9% ethanol at a ratio of 5:95 (v/v) was added to each tube, carefully mixed and then centrifuged at $2,100 \times g$ and 4°C for 30 min. Supernatants were collected into fresh tubes and diluted with gelatin-PBS to obtain the final solutions. The extracted standards and samples were stored at -20°C until analysis.

The final extracted solution (the supernatant diluted with gelatin-PBS) was then adjusted to 50 $\mu\text{l}/\text{tube}$, which was typically in the middle of the standard curve, for use in the RIA.

T4 RIA

For radioiodination, a tracer was prepared from a thyroxine-bovine serum albumin (BSA) conjugate (Cat. No. 8960, Bio-Rad AbD Serotec Limited, Raleigh, NC, U.S.A.) labeled with ^{125}I (NEZ033A, PerkinElmer, Inc., Waltham, MA, U.S.A.) using the previously described chloramine T method [19]. Total thyroxine (T4) concentrations were determined using a double-antibody RIA system. All samples and the standard (L-thyroxine, T2376, Sigma-Aldrich Co.) doses were measured in duplicate and triplicate

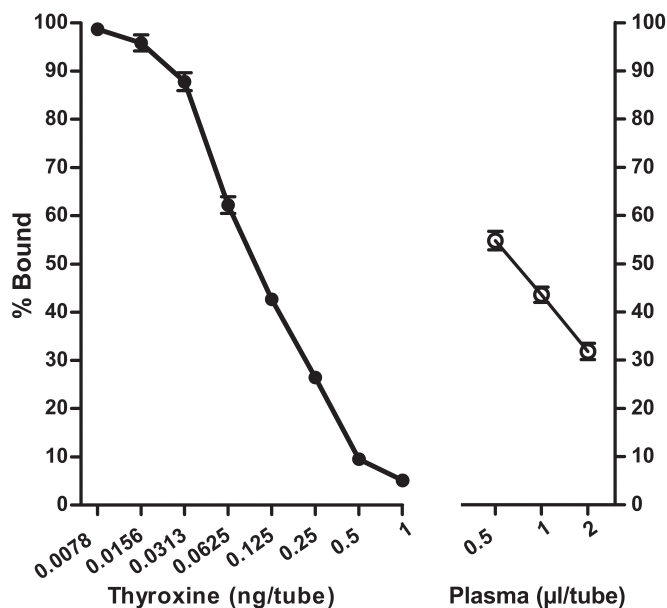


Fig. 1. Radioimmunoassay dose-response curves for total T4 concentrations using the sodium acetate ethanol method to separate binding proteins from plasma. T4 standards (●) from 0.0078 to 1 ng/tube and yearling plasma (○, n=10) using ¹²⁵I-labeled thyroxine as a tracer. The X-axis shows doses of T4 on a logarithmic scale. Values are expressed as means ± SEM of triplicate and duplicate measurements of standards and plasma samples, respectively. The percent bound from yearling plasma paralleled that of the T4 standard ($y = -2.06x - 0.34$ and $-2.17x - 2.35$, $r^2 = 0.773$ and 0.686 , respectively).

in the same assay. The diluted (1:1,000) primary polyclonal antibody (50 µl/tube), lyophilized rabbit anti-thyroxine BSA serum (Cat No. 65850, MP Biomedicals, LLC, Santa Ana, CA, U.S.A.) in modified GGB without sodium salicylate and containing 0.4% normal rabbit serum, was introduced into disposable polypropylene tubes (1.2 ml microtiter tubes for RIA, Thermo Scientific, San Diego, CA, U.S.A.). After mixing, all tubes were incubated at 4°C for 24 hr. Tracer T4 labeled with ¹²⁵I in modified GGB (50 µl; approximately 5,000 counts per min) was added, and the solution was mixed briefly. After incubation at 4°C for 24 hr, the diluted (1:40) secondary antibody (50 µl/tube), anti-rabbit-gamma-globulin (Veterinary Physiology Laboratory, Tokyo University of Agriculture and Technology) [18] in modified GGB containing 7% polyethylene glycol (Wako Pure Chemical Industries, Ltd.) was added to the tubes. After the solutions were mixed, the tubes were incubated at 4°C for 24 hr. Thereafter, bound and free ligands were separated by centrifugation at 1,700 ×g for 30 min at 4°C. The supernatant was decanted, and the precipitate was examined in a gamma counter (Cobra Quantum, PerkinElmer, Inc.) for 1 min. The intra-assay coefficient of variance was determined as previously described [7].

Statistical analysis

All results are expressed as means ± standard errors of the means (SEM). Logarithmically transformed dose concentrations were analyzed by linear regression to create the standard curve. Correlations between the sodium acetate ethanol and acid ethanol methods were analyzed by Spearman rank correlation. The differences in means of T4 values between horses raised in different climates and the repeated-measures data were analyzed using the generalized least-squares method [16, 36] with adjustments by Bonferroni's multiple comparison tests using R software. The level of significance was set at 0.05.

RESULTS

RIA performed with the sodium acetate ethanol method

Representative dose-response curves for the T4 standards and yearling horse plasma samples are shown in Fig. 1. Both the standard and samples were treated using the sodium acetate ethanol method. The standard curve showed linearity between the logarithmic doses of 0.0078 and 1 ng/tube. Dose-response curve for yearling horse samples at doses between 0.5 to 2 µl/tube paralleled the standard curve. The lowest dose of 0.5 µl/tube was close to 50% binding on the standard curve. The mean concentration of T4 in the 0.5 µl/tube was 225.98 ± 20.89 ng/ml. The intra-assay coefficient of variance was 5.78%, and assay sensitivity was 0.316 ng/ml.

Comparison of circulating T4 concentrations with the sodium acetate ethanol and acid ethanol methods

The percent bound using the two binding protein separation methods, the sodium acetate ethanol and acid ethanol methods, are

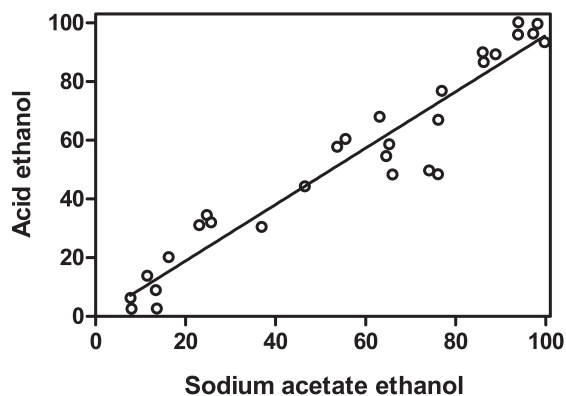


Fig. 2. Correlation of percent bound using the sodium acetate ethanol and acid ethanol methods for binding protein separation. Sodium acetate ethanol (n=30) and acid ethanol (n=30) methods with ¹²⁵I-labeled thyroxine as a tracer were analyzed in triplicate. A Spearman rank correlation analysis was conducted ($\rho=0.95$, $y=0.96x-0.29$, $P<0.0001$).

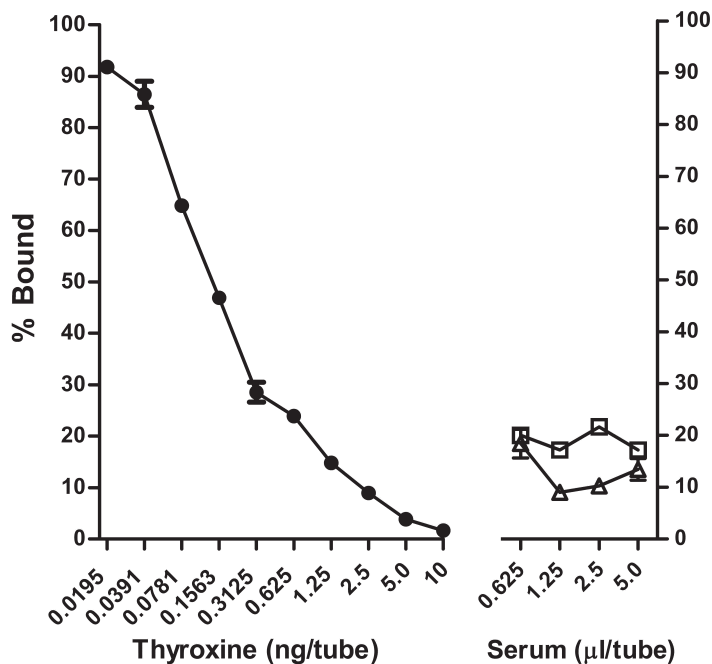


Fig. 3. Radioimmunoassay dose-response curves of total T4 concentrations in adult horse sera using the sodium salicylate method to separate binding proteins from serum. T4 standards (●) from 0.0195 to 10 ng/tube and estrus (□, n=1) and diestrus (Δ, n=1) sera using ¹²⁵I-labeled thyroxine as a tracer. Values are expressed as means ± SEM of triplicate and duplicate measurements from standard and serum samples.

shown in Fig. 2. There was a strong positive correlation between T4 concentrations determined using the sodium acetate ethanol and acid ethanol methods ($\rho=0.95$, $P<0.0001$).

RIA performed with the sodium salicylate method

To separate binding proteins using 2% sodium salicylate in the original GGB, standards and sera from adult horses were mixed with the original GGB to obtain the expected dose and were then diluted with the modified GGB to 0.3 and 0.6% sodium salicylate, respectively, for the assay. The representative dose-response curves for T4 showed linearity between the logarithmic doses of 0.0195 and 10 ng/tube. The dose-response curves for T4 in adult horse (horses in estrus or diestrus) sera did not parallel the standard curves. In fact, the percent bound from both estrous and diestrus sera was low at every dose and lower than those obtained with the sodium acetate ethanol method when compared at similar doses (Fig. 3). Furthermore, serial dilution of sodium salicylate in the original GGB showed that sodium salicylate affected the percent bound in a dose-dependent manner. However, the percent bound was constant from 0.016 to 0.002% sodium salicylate (Fig. 4). In addition, the use of 0.01% sodium salicylate for RIA resulted in measurements of very low levels of circulating T4 in horse samples (data not shown).

Comparison of circulating T4 concentrations between Hokkaido and Miyazaki yearlings using the sodium acetate ethanol method

To investigate whether the RIA using the sodium acetate ethanol method was an appropriate tool for determining T4 levels in horses, we obtained plasma and sera from yearling horses raised in different climates. Hokkaido, in the temperate north, and Miyazaki, in the subtropical south, Japan, were selected as sampling sites for this study. Samples were tested in duplicate after sodium acetate ethanol extraction. There were no significant differences in circulating T4 concentrations between male Hokkaido and Miyazaki yearlings throughout the experiment periods ($P>0.05$). Female Miyazaki yearlings, however, showed significantly lower T4 levels than those of female Hokkaido yearlings in late January ($P=0.007$). In addition, circulating T4 concentrations in Hokkaido yearlings of both sexes tended to be greater than those of Miyazaki yearlings of both sexes throughout the study period (Fig. 5A and 5B). The levels of circulating T4 were not significantly different by time of sampling in the Hokkaido and Miyazaki yearlings during the transition from winter to early spring ($P>0.05$).

DISCUSSION

Differences in the quantities and affinities of T4 binding proteins among species might influence the amount of total T4

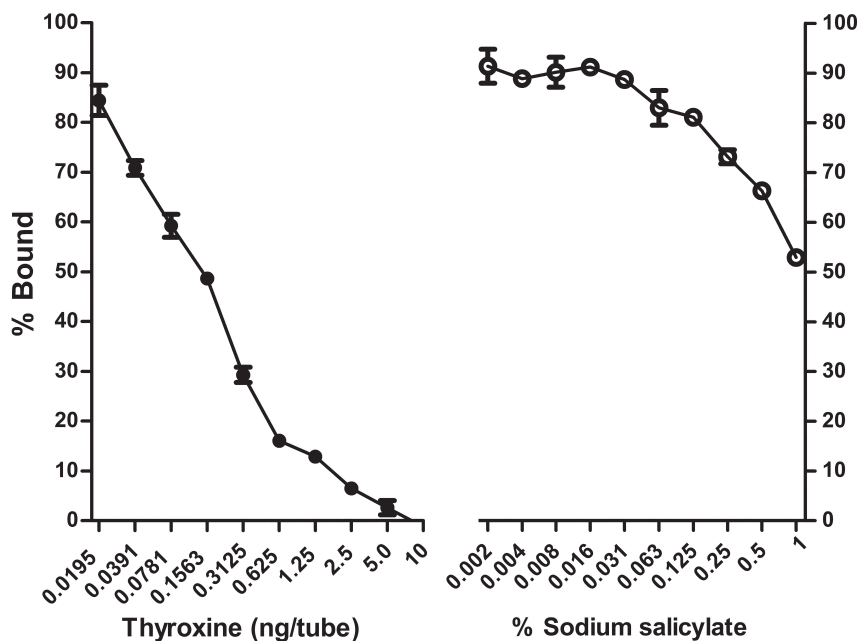


Fig. 4. Radioimmunoassay dose-response curves of total T4 concentrations using the sodium salicylate method to separate binding proteins from serum. T4 standards (●) from 0.0195 to 10 ng/tube and glycine gelatin buffer containing sodium salicylate (○) from 1% to 0.002% using ¹²⁵I-labeled thyroxine as a tracer. Values are expressed as means ± SEM of triplicate measurements from standard and glycine gelatin buffer.

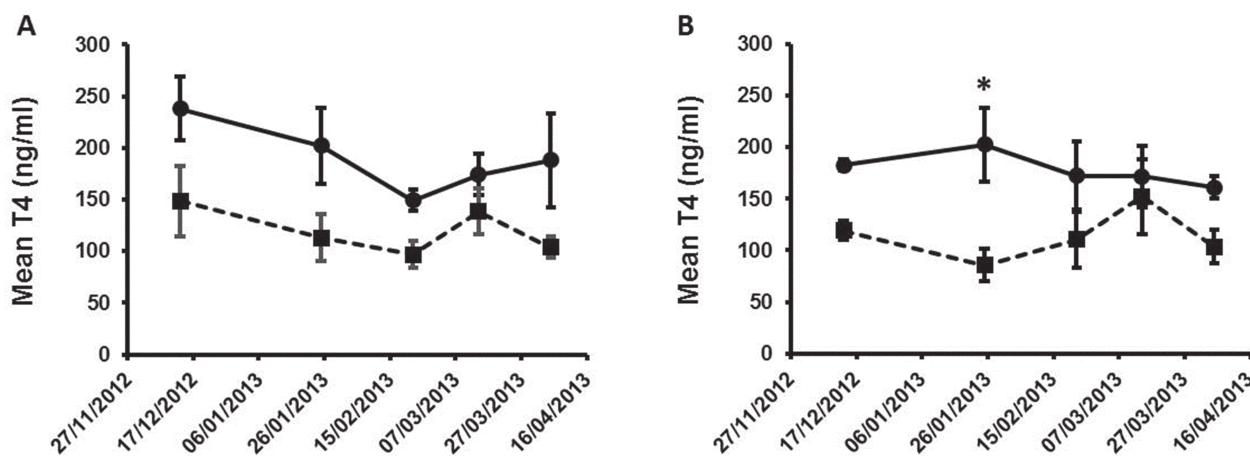


Fig. 5. Comparison of mean circulating total T4 concentrations between Hokkaido (●, n=10) and Miyazaki (■, n=10) yearlings, both colts (A) and fillies (B). Values are expressed as means ± SEM. *Denotes significant differences at $P < 0.05$ between horses in the two climate groups in each period and for each sex. A generalized least squares analysis with adjustment by Bonferroni's multiple comparison tests was performed.

levels in the blood circulation. Therefore, it is essential to remove any binding proteins in serum or plasma before performing immunoassays. In the present study, we adapted two extraction techniques, the sodium acetate ethanol and acid ethanol methods, and evaluated their suitability for determining equine T4 concentrations. The acid ethanol method, which has been commonly used for insulin-like growth factor I (IGF-1) measurement [6], requires several steps that increase the protocol time compared to that of the sodium acetate ethanol method. Therefore, we compared the efficiency of protein separation with the two modified methods. The present study revealed that the results of the sodium acetate ethanol method showed a strong positive correlation with those of the acid ethanol method (Fig. 2). This indicated that use of the modified sodium acetate ethanol method was not only similarly effective to acid ethanol for protein extraction but also simpler, especially with large sample quantities.

In previous attempts to measure thyroid hormones, sodium salicylate has been used as an inhibitor or displacer of serum proteins that bind to these thyroid hormones [35]. Several publications have shown that sodium salicylate is capable of binding plasma proteins in humans and other species [22, 32, 37] and that application of 2% sodium salicylate is required to separate binding proteins from serum. However, in our study, we found that 2% sodium salicylate markedly affected the binding of T4 radioligand

to primary antibody, resulting in a low percent bound from horse serum samples (Fig. 3). To avoid this effect, the sodium salicylate concentration should be low (e.g., 0.016%) (Fig. 4). However, dilution of sodium salicylate to 0.016% resulted in T4 levels that were too low for detection in horse serum samples (data not shown). For this reason, we used the alternative sodium acetate ethanol method for protein separation prior to RIA.

In the validation of our assay relative to that using the sodium acetate ethanol method, we found that the dose-response curves of yearling plasma paralleled those of the T4 standards (Fig. 1). In addition, we investigated and compared circulating T4 levels in yearling horses raised in different climatic conditions. Hokkaido, in the northern part of Japan, is located in a temperate zone; it had lower temperatures than those of Miyazaki in the subtropical south throughout the study period. In this study, there was a tendency for circulating T4 concentrations to be higher in Hokkaido yearlings than in Miyazaki yearlings, regardless of sampling time (Fig. 5). This result is consistent with that of previous research, in which horses housed in colder conditions tended to have higher T4 levels than those of horses housed in warmer climates during the winter [13, 24]. Our assay had a sensitivity and specificity that was sufficient for the evaluation of T4 concentrations reflecting physiologic responses to different climates.

We believe that determinations of total T4 levels are as important as those of fT4 levels in assessing thyroid gland function. This is supported by previous reports showing that fT4 concentrations in plasma are highly correlated with total T4 concentrations [3, 8, 38]. Interestingly, total T4 levels in yearlings obtained in our RIA, 225.98 ± 20.89 ng/ml, were dramatically different from the reference values of 35 ng/ml [27] and 68.68 ± 2.0 ng/ml in horses at 6 months to 1 year of age, or 34.61 ± 0.92 ng/ml in horses 1–3 years of age [17]. This suggested that the modified sodium acetate ethanol method used in our study was capable of separating binding proteins, but did not affect binding between the antibody and T4. In addition, our results revealed circulating T4 concentrations that were higher than those of previous reports [17, 27]. This may indicate that our immunoassay was more physiologically appropriate for separating binding protein from serum and plasma, allowing the true value of total T4 in equine species to be revealed. Because of variations in plasma proteins between animals, further studies of T4 in other species should be conducted.

In conclusion, this is the first report describing a modified sodium acetate ethanol technique that is simple and convenient for separating T4-binding protein from serum and plasma, and the first study to establish a radioimmunoassay specifically to measure circulating total T4 in horses. We recommend that T4 levels associated with other physiologic changes be investigated to more fully understand thyroid gland function in yearling horses.

ACKNOWLEDGMENTS. We are thankful to all staff members of the Hidaka Training and Research Center in Hokkaido and Miyazaki Training Yearling Farm in Miyazaki, both members of the Japan Racing Association, for collecting horse blood samples. We express our sincere gratitude to Dr. Reinhold J. Hutz (Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI, U.S.A.) for reading the original manuscript and for valuable suggestions.

REFERENCES

1. Bird, J. A., Clarke, L. and Symonds, M. E. 1998. Influence of thyrotrophin-releasing hormone on thermoregulation in newborn lambs. *Biol. Neonate* **73**: 52–59. [Medline] [CrossRef]
2. Breuhaus, B. A., Refsal, K. R. and Beyerlein, S. L. 2006. Measurement of free thyroxine concentration in horses by equilibrium dialysis. *J. Vet. Intern. Med.* **20**: 371–376. [Medline] [CrossRef]
3. Brinkmann, L., Gerken, M., Hambly, C., Speakman, J. R. and Riek, A. 2016. Thyroid hormones correlate with field metabolic rate in ponies, *Equus ferus caballus*. *J. Exp. Biol.* **219**: 2559–2566. [Medline] [CrossRef]
4. Chen, C. L. and Riley, A. M. 1981. Serum thyroxine and triiodothyronine concentrations in neonatal foals and mature horses. *Am. J. Vet. Res.* **42**: 1415–1417. [Medline]
5. Chopra, I. J., Sack, J. and Fisher, D. A. 1975. 3,3',5'-Triiodothyronine (reverse T3) and 3,3',5-triiodothyronine (T3) in fetal and adult sheep: studies of metabolic clearance rates, production rates, serum binding, and thyroidal content relative to thyroxine. *Endocrinology* **97**: 1080–1088. [Medline] [CrossRef]
6. Daughaday, W. H., Mariz, I. K. and Blethen, S. L. 1980. Inhibition of access of bound somatomedin to membrane receptor and immunobinding sites: a comparison of radioreceptor and radioimmunoassay of somatomedin in native and acid-ethanol-extracted serum. *J. Clin. Endocrinol. Metab.* **51**: 781–788. [Medline] [CrossRef]
7. Dudley, R. A., Edwards, P., Ekins, R. P., Finney, D. J., McKenzie, I. G., Raab, G. M., Rodbard, D. and Rodgers, R. P. 1985. Guidelines for immunoassay data processing. *Clin. Chem.* **31**: 1264–1271. [Medline]
8. Elliott, K. H., Welcker, J., Gaston, A. J., Hatch, S. A., Palace, V., Hare, J. F., Speakman, J. R. and Anderson, W. G. 2013. Thyroid hormones correlate with resting metabolic rate, not daily energy expenditure, in two charadriiform seabirds. *Biol. Open* **2**: 580–586. [Medline] [CrossRef]
9. Engelking, L. R. 2002. Review of Veterinary Physiology, Teton NewMedia, Wyoming.
10. Eshratkhan, B., Rajabian, H., Namvar, D., Eshratkhan, S. and Bastam, S. M. 2011. Comparative study on determination of plasma thyroid hormones by chemiluminescence and electrochemiluminescence immunoassay methods in sheep. *Comp. Clin. Pathol.* **20**: 135–138. [CrossRef]
11. Esquivel, M. S. and Ramirez, L. C. 2016. Measurement of thyroid hormones and cortisol in horses with an automated immunoassay analyzer. *Rev. Ciencias Veterinarias* **34**: 39–49.
12. Evinger, J. V. and Nelson, R. W. 1984. The clinical pharmacology of thyroid hormones in the dog. *J. Am. Vet. Med. Assoc.* **185**: 314–316. [Medline]
13. Fazio, E., Medica, P., Cravana, C. and Ferlazzo, A. 2012. Total and free iodothyronines profile in the donkey (*Equus asinus*) over a 12-month period. *Acta Vet. Brno* **81**: 239–244. [CrossRef]
14. Fitzgerald, B. P. and Davison, L. A. 1998. Thyroxine concentrations are elevated in mares which continue to exhibit estrous cycles during the nonbreeding season. *JEVs* **18**: 48–51.
15. Graves, E. A., Schott, H. C. 2nd., Marteniuk, J. V., Refsal, K. R. and Nachreiner, R. F. 2006. Thyroid hormone responses to endurance exercise.

- Equine Vet. J. Suppl.* **36**: 32–36. [Medline] [CrossRef]
16. Gueorguieva, R. and Krystal, J. H. 2004. Move over ANOVA: progress in analyzing repeated-measures data and its reflection in papers published in the Archives of General Psychiatry. *Arch. Gen. Psychiatry* **61**: 310–317. [Medline] [CrossRef]
 17. Gupta, A. K., Kumar, S. and Pal, Y. 2002. Biochemical, haematological and thyroid hormone profile in healthy Indian Kathiawari horses. *Asian-Aust. J. Anim. Sci.* **15**: 1215–1221.
 18. Hamada, T., Watanabe, G., Kokuho, T., Taya, K., Sasamoto, S., Hasegawa, Y., Miyamoto, K. and Igarashi, M. 1989. Radioimmunoassay of inhibin in various mammals. *J. Endocrinol.* **122**: 697–704. [Medline] [CrossRef]
 19. Hasegawa, Y., Miyamoto, K., Fukuda, M., Takahashi, Y. and Igarashi, M. 1986. Immunological study of ovarian inhibin. *Endocrinol. Jpn.* **33**: 645–654. [Medline] [CrossRef]
 20. Huszenicza, G., Nagy, P., Juhász, J., Kóródi, P., Kulcsár, M., Reiczigel, J., Guillaume, D., Rudas, P. and Solti, L. 2000. Relationship between thyroid function and seasonal reproductive activity in mares. *J. Reprod. Fertil. Suppl.* **56**: 163–172. [Medline]
 21. Kadunc, N. C., Kosec, M. and Cestnik, V. 2003. Serum Triiodothyronine (T3) and Thyroxine (T4) concentrations in Lipizzan horses. *Acta Vet. Brno* **72**: 17–22. [CrossRef]
 22. McArthur, J. N. and Smith, M. J. H. 1968. Binding of salicylate to albumin. *FEBS Lett.* **1**: 332–334. [Medline] [CrossRef]
 23. Medica, P., Fazio, E., Cravana, C. and Ferlazzo, A. 2011. Influence of endemic goitre areas on thyroid hormones in horses. *Animal* **5**: 82–87. [Medline] [CrossRef]
 24. Mejdell, C. M. and Bøe, K. E. 2005. Responses to climatic variables of horses housed outdoors under Nordic winter conditions. *Can. J. Anim. Sci.* **85**: 307–308. [CrossRef]
 25. Messer, N. T. 1993. Clinical and diagnostic features of thyroid disease in horses. Proc 11th ACVIM Forum, Washington, DC: 649–651.
 26. Nomura, Y., Abe, Y., Ishii, Y., Watanabe, M., Kobayashi, M., Hattori, A. and Tsujimoto, M. 2003. Structural changes in the glycosaminoglycan chain of rat skin decorin with growth. *J. Dermatol.* **30**: 655–664. [Medline] [CrossRef]
 27. Reed, S. M., Bayly, W. M. and Sellon, D. C. 2004. *Equine Internal Medicine*, 2nd ed., W.B. Saunders Co., St. Louis.
 28. Singh, A. K., Jiang, Y., White, T. and Spassova, D. 1997. Validation of nonradioactive chemiluminescent immunoassay methods for the analysis of thyroxine and cortisol in blood samples obtained from dogs, cats, and horses. *J. Vet. Diagn. Invest.* **9**: 261–268. [Medline] [CrossRef]
 29. Slebodziński, A. B., Brzezińska-Slebodzińska, E., Nowak, J. and Kowalska, K. 1998. Triiodothyronine (T3), insulin and characteristics of 5'-monodeiodinase (5'-MD) in mare's milk from parturition to 21 days post-partum. *Reprod. Nutr. Dev.* **38**: 235–244. [Medline] [CrossRef]
 30. Sojka, J. E., Johnson, M. A. and Bottoms, G. D. 1993. Serum triiodothyronine, total thyroxine, and free thyroxine concentrations in horses. *Am. J. Vet. Res.* **54**: 52–55. [Medline]
 31. Sophianopoulos, J., Jerkunica, I., Lee, C. N. and Sgoutas, D. 1980. An improved ultrafiltration method for free thyroxine and triiodothyronine in serum. *Clin. Chem.* **26**: 159–162. [Medline]
 32. Sturman, J. A. and Smith, M. J. H. 1967. The binding of salicylate to plasma proteins in different species. *J. Pharm. Pharmacol.* **19**: 621–623. [Medline] [CrossRef]
 33. Todini, L., Malfatti, A., Salimei, E. and Fantuz, F. 2010. Measurement of thyroid hormones in donkey (*Equus asinus*) blood and milk: validation of ELISA kits and evaluation of sample collection, handling and storage. *J. Dairy Res.* **77**: 419–424. [Medline] [CrossRef]
 34. Todini, L., Salimei, E., Malfatti, A., Brunetti, V. L. and Fantuz, F. 2015. Thyroid hormones in donkey blood and milk: Correlations with milk yield and environmental temperatures. *Ital. J. Anim. Sci.* **14**: 4089. [CrossRef]
 35. Tohei, A., Akai, M., Tomabechi, T., Mamada, M. and Taya, K. 1997. Adrenal and gonadal function in hypothyroid adult male rats. *J. Endocrinol.* **152**: 147–154. [Medline] [CrossRef]
 36. Ugrinowitsch, C., Fellingham, G. W. and Ricard, M. D. 2004. Limitations of ordinary least squares models in analyzing repeated measures data. *Med. Sci. Sports Exerc.* **36**: 2144–2148. [Medline] [CrossRef]
 37. Wang, R., Nelson, J. C. and Wilcox, R. B. 1999. Salsalate and salicylate binding to and their displacement of thyroxine from thyroxine-binding globulin, transthyrin, and albumin. *Thyroid* **9**: 359–364. [Medline] [CrossRef]
 38. Welcker, J., Chastel, O., Gabrielsen, G. W. G., Guillaumin, J., Kitaysky, A. S., Speakman, J. R., Tremblay, Y. and Bech, C. 2013. Thyroid hormones correlate with basal metabolic rate but not field metabolic rate in a wild bird species. *PLoS ONE* **8**: e56229. [Medline] [CrossRef]
 39. Wheeler, M. J. 2013. A short history of hormone measurement. *Methods Mol. Biol.* **1065**: 1–6. [Medline] [CrossRef]