A High-Performance Liquid-Chromatographic Analysis of Serum Tocopherols in Thoroughbred Horses

Hiromasa WATANABE,* Yoshikazu Fujii,* Fumikatsu ICHIKAWA,* Katsuhiro NIWA** and Takeshi YAMAMOTO**

A study was carried out on a high-performance liquid-chromatographic (HPLC) analysis of horse serum tocopherols to explore a part of the etiology of the muscle diseases in the Thoroughbred horse. As a result, linear standard calibration curves, high sensitivity and reproducibility, and a recovery of over 95% of the tocopherols were obtained. Overlapping findings between d-α-tocopherol standard and d-α-tocopherol of horse serum were observed in the chromatogram. The serum α-tocopherol level in Thoroughbred horses was evidently lower than, or about 1/4 to 1/3 of that in human beings. The difference in α-tocopherol level was significant, at P<0.05, between the Thoroughbred racehorse and the Thoroughbred riding horse. Regarding the above mentioned overlapping finding of tocopherol, the peaks of β-tocopherol in horse serum and denatured vitamin A possessed the same retention time. The peak of the former could not be estimated separately from that of the latter by HPLC analysis with the UV detector alone. In racehorses the mean serum α-tocopherol level in the post-exercise pre-feeding in the early morning was lower than the level in the post-feeding resting time. The serum α-tocopherol level was examined in 7 horses with the tying-up syndrome 7–10 days after attack. It was lower in 3 horses, but was not so much lower in the other 4 horses than in the healthy racehorses.

Introduction

To contribute to the accurate clinical diagnosis of muscle diseases in the horse, studies had been performed on the diagnostic values of serum enzymes, isoenzymes,1–5) and myoglobin.6,7) No etiology of these diseases in the horse, however, has been clarified. In some other animal species, it was reported that deficiency in vitamin E or selenium was the cause of muscle diseases.8,9) A few findings were reported on deficiency in total serum vitamin E in horses.10,11) No information is available on isomeric tocopherols in the horse. In the horse, no intimate relationship has been shown between deficiency in tocopherols and muscle diseases.

It was reported that fat-soluble substances other than tocopherols in the human serum sometimes interfered with the determination by commonly used methods, but did not with that by HPLC
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analysis. It was also reported that each isomeric tocopherol possessed extremely different biological activity, and that HPLC analysis was the most efficient of the methods for determination of human serum tocopherols. Therefore, in this study, HPLC analysis was employed for determination of horse serum tocopherols.

The muscle disease has presented a high incidence among the disease of the locomotor system in the racehorses in Japan. For the purpose of elucidation of the etiology of muscle disease, the present investigation, as the first step, deals with the serum \( \alpha \)-tocopherol level in the Thoroughbred horse.

**Materials and Methods**

Tocopherols in the horse serum were determined by the following apparatus: A high-performance liquid-chromatographic system (Waters Associates, Milfold, Massachusetts 07157 USA) consisting of a solvent-delivery system Model 3000, universal injector, Model U6K, and ultraviolet (UV) absorbance detector, Model 440. The detector was operated at 280 nm at a sensitivity of 0.05 scale.

Moreover, a high-polarity \( \mu \)-Bondapak NH\(_2\) column, 300 \( \times \) 3.9 mm, was used.

The mobile phase was isopropanol/n-hexane (5/95 by vol.) and the flow rate at 0.5 ml/min (at a pressure of about 200 psi.). The chart speed of the recorder was 5 mm/min.

Tocopherols in the horse serum were extracted with distilled water, ethanol, and n-hexane by the method of Abe and Katsui, as shown in Table 1. The n-hexane layer was evaporated completely in a bath at 40\(^\circ\)C under a stream of nitrogen, and the residue dissolved in a small amount of n-hexane. A part of the aliquot was injected into HPLC.

The concentration of serum \( \alpha \)-tocopherol was calculated from the height of a peak in the chromatogram on the basis of the standard calibration curve and recovery rate (Fig. 2). The d-tocopherol standard and dl-tocol for internal standard were supplied from the Eisai Research Institute by the courtesy of Drs. Katsui, Abe and Ohkubo.

Serum samples were collected from the jugular vein into evacuated blood collection tubes (Vacutainer, Becton-Dickinson, Rutherford, New Jersey 07070, USA). The tubes were covered with aluminum foil to block up sun light. The samples were placed in vials covered with the same foil and stored at \(-20^\circ\)C. Determination of tocopherols was carried out within 2 months.

The Thoroughbred racehorse and rearing and riding Thoroughbred horse used in this investigation were clinically healthy males and females ranging from 2 to 6 years of age. They had been stabled at the Miho Training Center, at the Utsunomiya Rearing Farm, and at the Eques-
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trian Park, Japan Racing Association. Further, serum \( \alpha \)-tocopherol level was determined in samples collected from 5 experimental Thoroughbred horses before and after exercises and 7 Thoroughbred racehorses with the tying-up syndrome.

**Results**

In order to select the best fractional condition, the proportions of mixture ranging from 2:98 to 8:92 of isopropanol and n-hexane were examined. A 5:95 mixture by volume was the most suitable for the mobile phase of this HPLC.

The peak height of HPLC was directly proportional to the injected amount of the standard solution of tocopherols, as demonstrated in Fig. 1. The retention times of d-\( \alpha \)-, d-\( \beta \)-, and d-\( \delta \)-tocopherol by the chromatography were nearly 9 min 40 s, 12 min 20 s, and 16 min 10 s, respectively. Fig. 2 shows linear standard calibration curves obtained by chromatography.

In HPLC, the sensitivity, reproducibility in the estimation on horse serum, and rate of recovery of tocopherols obtained by the method of standard addition were high. The coefficient of variation (CV) of the serum sample was less than 4.0\% \((n=24)\). The rate of recovery of tocopherols in the serum was over 95\% \((n=8)\).

Fig. 3 shows the chromatogram of horse serum sample obtained by HPLC.

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**Fig. 1.** Chromatograms of tocopherols standard for calibration curve

Remarks. *: Injected amount of tocopherols standard. tocoph.: tocopherol.

**Fig. 2.** Standard calibration curves for the determination of serum tocopherol level

**Fig. 3.** Overlapping finding of chromatograms in serum \( \alpha \)-tocopherol and \( \alpha \)-tocopherol standard

Remarks. Arrows indicate the peaks of \( \alpha \)-tocopherol. Left chromatogram: Extract from horse serum. Right chromatogram: Extract from the same serum to which was added \( \alpha \)-tocopherol standard.
It also presents the chromatogram of the same sample to which had been added a small amount of d-α-tocopherol standard. This chromatogram demonstrated the overlapping finding of α-tocopherol standard and that in horse serum. Regarding β-tocopherol, the same overlapping finding of the chromatogram was found in the standard and in serum sample, as shown in Fig. 4.

When studies were made on the method of HPLC analysis of vitamin A in the horse serum, the retention time of β-tocopherol, which was nearly 12 min 20 s, was the same as that of denatured vitamin A.

The peak of α-tocopherol was found in each horse serum sample. The peak of β-tocopherol, however, was observed in some samples, but not in others, as shown in Fig. 5. The same finding was obtained from δ-tocopherol in the horse serum, although the peak exhibited no overlapping with that of denatured vitamin A. Further, one of unknown peaks was observed in many samples, immediately following the peak of vitamin A (Fig. 5).

The mean α-tocopherol level in the serum was 2.6 µg/ml in the healthy Thoroughbred racehorses, and was 2.3 and 3.3 µg/ml in the rearing and the riding horses, respectively, when determined in samples collected over a period from 9 to 11 a.m., or at the time of resting in the stable after feeding (Table 2).

On the other hand, the α-tocopherol level in serum was low, or 1.9 µg/ml,
in the healthy Thoroughbred racehorses, except eleven which were 3 years old, in the pre-feeding post-exercise early morning (Table 3). In 5 experimental

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<th>Table 2. Serum α-tocopherol level in Thoroughbred horses</th>
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<tbody>
<tr>
<td>Age in years n</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Racehorse 2 13</td>
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<tr>
<td>3–5 14</td>
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<tr>
<td>Total (2–5) 27</td>
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<tr>
<td>Rearing horse 2 31</td>
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<td>Riding horse 3–6 22</td>
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Remarks. *: Standard deviation

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<th>Table 3. Serum α-tocopherol level in Thoroughbred racehorses after exercise</th>
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<td>Age in years n</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>2 40</td>
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<tr>
<td>3 11</td>
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<tr>
<td>4–6 11</td>
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<td>Total (2–6) 62</td>
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Remarks. *: Standard deviation

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<th>Table 4. Serum α-tocopherol level in experimental Thoroughbred horses before and after exercise</th>
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<td>Time of blood collection</td>
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<tr>
<td>Before exercise 2 5 2.72±1.01</td>
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<tr>
<td>Immediately after exercise 2.78±0.86</td>
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Remarks. *: Standard deviation

Thoroughbred horses, the serum α-tocopherol level was not reduced by the exercise, as shown in Table 4, but the finding of blood concentration was presented by the change of the serum protein level.

There was no significant difference in the mean serum α-tocopherol level between the racehorses and the rearing horses. The difference in this level was significant, at P<0.05, between the racehorses and the riding horses and between the rearing and the riding horses. Fig. 6 illustrates mean α-tocopherol levels in serum in the Thoroughbred horse.

The α-tocopherol level in serum samples from 7 Thoroughbred racehorses with the tying-up syndrome was demonstrated in Fig. 7. It was lower in 3 horses and a little lower in the other four than in healthy racehorses.

Fig. 6. Serum α-tocopherol level in Thoroughbred horses

Remarks. *: Samples from period of resting at stable after feeding, or from 9 to 11 a.m.

**: Samples from period of post-exercise, pre-feeding, in the early morning.

***: years old.
Fig. 7. Chromatograms of serum in racehorses with normal and low α-tocopherol level
Remarks. Left: Chromatogram of horse serum with a normal α-tocopherol level of 2.6 μg per ml.
Right: Chromatogram of horse serum with a low α-tocopherol level of 0.9 μg per ml. Of 7 cases of the tying-up syndrome, the level was 0.9–1.6 μg per ml in 3 cases, and 1.8–2.7 μg per ml in the other 4 cases.

Discussion

Peaks of fat-soluble substances other than tocopherols and vitamin A, which had a short retention time, were observed by HPLC. Similar findings have been seen in the report of HPLC analysis on human serum tocopherols with a fluorescence detector. Those substances could be distinguished by HPLC on the basis of the difference in retention time. In the analysis of total tocopherol level by the conventional spectrophotometry, however, contamination of these substances was assumed to be inevitable.

Peaks of the same retention time as that of β-tocopherol standard could not be identified whether they were of β-tocopherol or of denatured vitamin A. The identification of this peak is now studied. Besides, the peak immediately following that of vitamin A must be identified in future.

It was reported that α-tocopherol possessed the highest biological activity, and that this tocopherol was the most important for the human and animal organism of the isomeric tocopherols. There still remain, however, some problems on unknown peaks, as above mentioned. HPLC with UV of horse serum tocopherols is presumed to be efficient, since it possesses an advantage of conducting the direct determination of the most important α-tocopherol. HPLC is also presumed to be useful for the analysis of serum samples with a low level of α-tocopherol, because of its high sensitivity, recovery rate, and reproducibility. Nevertheless, the analysis of horse serum tocopher-
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erols by using HPLC with UV and fluorescent detector is assumed to be more advantageous because of its specific spectrophotometric properties.\textsuperscript{12,15} A peak of $\delta$-tocopherol was observed in some horse sera. It did not seem to be important for the animal organism, because of its very low biological activity.\textsuperscript{14,15}

The serum $\alpha$-tocopherol level in healthy Thoroughbred horses was much lower than, or about 1/4 to 1/3 of, that in human beings.\textsuperscript{17} This result is assumed to be partly due to the difference in uptakes of both tocopherol and unsaturated fatty acids. The difference in serum $\alpha$-tocopherol level between the racehorse and the riding horse is considered to be derived from the difference in the quality and quantity of exercise loading.

From the results obtained from the horses with the tying-up syndrome, deficiency or disturbance in intestinal absorption of tocopherol is assumed to be one of the causative factors of the muscle disease in question. A more adequate investigation on deficiency in tocopherols is essential. It is actually performed at present. Concerning the features of tocopherols on the antioxidant and stabilizing effect of the biomembrane, it is known that this vitamin act upon many systemic organs.\textsuperscript{8,18,19} Then, it seems necessary to carry out studies on diseases, other than muscle diseases, caused by deficiency of this vitamin.

Vitamin A is a very important nutrient,\textsuperscript{20} but it inhibits\textsuperscript{19} the effect of vitamin E of stabilizing the biomembrane.\textsuperscript{21,22} Therefore, a study is now under way on vitamin A in the horse serum by means of HPLC analysis. Further, an investigation will be performed on deficiency of disturbance in the intestinal absorption of selenium, which is a constituent element of glutathione peroxidase.

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