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# Short Communication

# Simultaneous determination of retinol and tocopherols by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method to determine retinol and all four tocopherols (α-, β-, γ- and δ-) simultaneously was established using a reversed-phase column (YMC-PACK A-302 S-5 120A ODS). The HPLC conditions were mobile phase 65% isopropanol, sample solvent 99.5% methanol and temperature 30°C. Retinol and tocopherols were measured in rat liver.

## INTRODUCTION

Reports on the simultaneous measurement of retinol and tocopherols by high-performance liquid chromatography (HPLC) using reversed-phase columns have been presented by several groups [1–4]. However, with these methods tocopherols can be separated into only three fractions, *i.e.*, α-, β- + γ- and δ-tocopherols. Vatassery *et al.* [5] reported that the separation of β- and γ-tocopherols using reversed-phase columns is difficult. Although Wahyuni and Jinno [6] succeeded, the separation was inadequate and the elution time was over 2 h.

Using the reversed-phase HPLC procedure described in this paper, the separation of all four tocopherols and retinol was clearly achieved within 1 h. Retinol and tocopherols were measured in rat liver using this method.

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## EXPERIMENTAL

### *Apparatus*

The following instruments (all obtained from Shimadzu, Kyoto, Japan) were used; HPLC pump, Model LC-3A; sample injector, Model LC-1; detector RF-500 spectrofluorometer (for retinol, $\lambda_{ex}$ 340 nm, $\lambda_{em}$ 460 nm; for tocopherol, $\lambda_{ex}$ 298 nm, $\lambda_{em}$ 325 nm); and recorder and computer, Chromatopac C-R6A.

A YMC-PACK A-302 S-5 120A ODS reversed-phase column (150 mm × 4.6 mm I.D. silica gel particle diameter 5 μm, pore size 120 Å, with residual silane groups end-capped with trimethylsilyl chloride, was kindly donated by YMC (Kyoto, Japan).

### *Reagents*

*d*-α-, -β-, -γ- and -δ-tocopherols were kindly donated by Eisai (Tokyo, Japan). Retinol was obtained from Fluka (Buchs, Switzerland). Ethanol,

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*n*-hexane, methanol and 2-propanol were of HPLC grade from Nacalai Tesque (Kyoto, Japan).

As mobile phases, several mixtures of ethanol, methanol, 2-propanol and deionized, distilled water were examined.

*Procedure*

Standard solutions of the four tocopherols and retinol were prepared in 99.5% methanol, 50.0% methanol, 99.5% ethanol and 99.5% 2-propanol. They were injected directly into the column.

*Preparation of sample*

Under diethyl ether anaesthesia, blood was drawn from the abdominal aorta of 9-week-old rats. The livers were then collected. Tocopherols and retinol were extracted from the livers by the method of Abe and Katsui [7].

## RESULTS

*Mobile phase and temperature*

First, HPLC was performed at ambient temperature (*ca.* 24°C) and 99.5% methanol was used as the sample solvent.

It was possible to determine retinol and three fractions of tocopherols (α-, β- + γ, δ-) using the mobile phases containing from 5% to 20% of water in methanol. However, β- and γ-tocopherols were not separated using methanol–water mobile phases.

It was found that β- and γ-tocopherols were hardly separated using mobile phases containing from 5% to 13% of water in ethanol. With over 14% of water in ethanol in the mobile phase, β- and γ-tocopherols could be separated, but the separation was insufficient. By further increasing the water content in ethanol, the difference in retention time between β- and γ-tocopherol was increased. With 20% of water in ethanol as the mobile phase, the time delay between β- and γ-tocopherols was 2.7 min. However, as the content of water in the mobile phase was increased, the retention times of the peaks became longer.

With mobile phases containing more than 15% of water in 2-propanol it was possible to separate β- and γ-tocopherols better than with other mobile phases, and a clear separation was obtained with 30% of water. However, it was found that the separation of β- and γ-tocopherols was incomplete when the ambient temperature was over 25°C. Even below 24°C, unstable temperatures resulted in unstable retention times. In order to obtain the optimum retention time, a constant column temperature was found to be necessary, which was controlled by a column oven. From 30 to 40°C, separation was performed using mobile phases containing from 60% to 70% of 2-propanol. When the 2-propanol content was 60% at 35°C, the maximum resolution ($R_s$) [8] was obtained (Table I). However, it took 74.7 min to elute all the tocopherols. At

TABLE I

PEAK RESOLUTION ($R_s$) OF β- AND γ-TOCOPHEROLS UNDER DIFFERENT HPLC CONDITIONS

| Mobile phase | Sample solvent | Temperature (°C) | Flow-rate (ml/min) | Retention time of α-tocopherol (min) | $R_s$ |
|---|---|---|---|---|---|
| 80% ethanol | 99.5% methanol | Ambient (24) | 0.7 | 102.7 | 0.89 |
| 70% 2-propanol | 99.5% methanol | Ambient (24) | 0.7 | 49.8 | 1.24 |
| 70% 2-propanol | 99.5% methanol | 30 | 0.7 | 28.5 | 0.89 |
| 70% 2-propanol | 99.5% methanol | 35 | 0.7 | 25.1 | 0.86 |
| 65% 2-propanol | 99.5% methanol | 30 | 0.7 | 48.9 | 1.26 |
| 65% 2-propanol | 99.5% methanol | 35 | 0.7 | 44.4 | 1.07 |
| 65% 2-propanol | 99.5% methanol | 40 | 0.7 | 41.2 | 0.89 |
| 60% 2-propanol | 99.5% methanol | 30 | 0.7 | 88.8 | 1.22 |
| 60% 2-propanol | 99.5% methanol | 35 | 0.7 | 74.7 | 1.28 |
| 60% 2-propanol | 99.5% methanol | 40 | 0.7 | 67.7 | 1.13 |
| 65% 2-propanol | 99.5% ethanol | 30 | 0.7 | 49.2 | 0.96 |
| 65% 2-propanol | 99.5% 2-propanol | 30 | 0.7 | 48.0 | 0.60 |
| 65% 2-propanol | 65.0% 2-propanol | 30 | 0.7 | 47.5 | 1.10 |
| 65% 2-propanol | 50.0% methanol | 30 | 0.7 | 47.7 | 1.22 |

30°C, using 65% of 2-propanol in the mobile phase, the resolution $R_s$ was 1.26, and all the tocopherols were eluted within 50 min (Table I).

*Sample solvent*

As sample solvents, 99.5% ethanol, 99.5% 2-propanol and 99.5% methanol were compared, while employing a mobile phase containing 65% of 2-propanol at 30°C. The best peak separation was obtained with 99.5% methanol (Table I). Then 99.5% methanol was compared with 50% methanol under the same conditions as described above. No difference was found concerning the separation of β- and γ-tocopherols (Table I). To dissolve the sample easily, 99.5% methanol was adopted as the solvent.

The most suitable conditions for the determination of retinol and all four tocopherols are shown in Table II. An elution profile for a standard solution obtained under these conditions is shown in Fig. 1A.

*Sensitivity*

The sensitivity of the method was calculated from the differences between duplicates using the method of Brown *et al.* [9]. The detection limits were 10 ng of α-tocopherol, 5 ng of β-, γ- and δ-tocopherols and 60 pg of retinol.

*Quantification*

There was a linear relationship between peak area and the amount of each compound injected ranging from 0.1 to 2.0 μg. The equations for retinol and α-, β-, γ- and δ-tocopherols were $y = 6.29x$, $y = 1.76x$, $y = 3.14x$, $y = 3.25x$ and $y = 2.84x$, respectively, where $y$ = peak area ($\times 10^4$) and $x$ = amount of compound (μg).

TABLE II

HPLC CONDITIONS FOR SIMULTANEOUS DETERMINATION OF RETINOL AND TOCOPHEROLS AFTER COMPLETE SEPARATION

| | |
|---|---|
| Column | YMC-PACK A-302 S-5 120A ODS (150 mm × 4.6 mm I.D.) |
| Mobile phase | 65% 2-propanol |
| Sample solvent | 99.5% methanol |
| Detector | Spectrofluorometer<br>For retinol: $\lambda_{ex}$ 340 nm, $\lambda_{em}$ 460 nm<br>For tocopherols: $\lambda_{ex}$ 298 nm, $\lambda_{em}$ 325 nm |
| Flow-rate | 0.7 ml/min |
| Temperature | 30°C |
| Column pressure | 140 kg/cm² |

*Determination of retinol and tocopherols in rat liver*

Fig. 1B shows an elution profile of retinol and tocopherols in a rat liver sample. The mean extraction recoveries ± standard error ($n$ = 10) were retinol 100.3 ± 3.1, α-tocopherol 106.1 ± 1.8, β-tocopherol 98.1 ± 2.9, γ-tocopherol 98.2 ± 3.4 and δ-tocopherol 96.3 ± 2.8%.

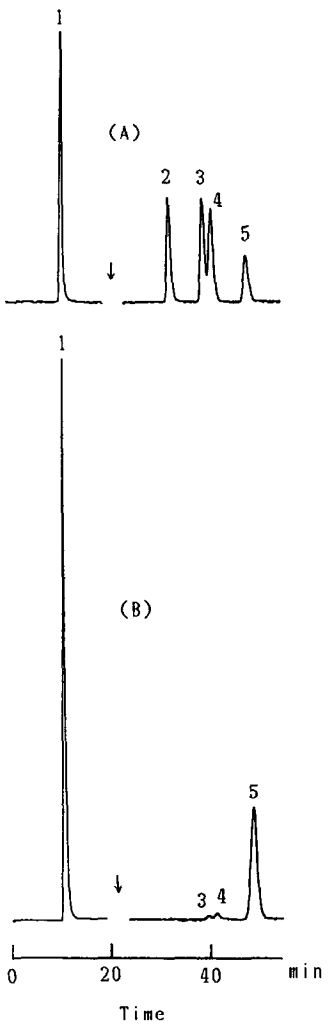


Fig. 1. Chromatograms of retinol and tocopherols. (A) Standard solution; (B) sample of rat liver. Conditions as in Table II. Peaks: 1 = retinol; 2 = δ-tocopherol; 3 = γ-tocopherol; 4 = β-tocopherol; 5 = α-tocopherol. Arrows indicate change of wavelength.

## DISCUSSION

The effects of vitamin E on vitamin A metabolism have been reported in a number of papers [10–13]. Many physicians recommend concomitant treatment with vitamin A when patients suffering from abetalipoproteinaemia are treated with vitamin E [14,15]. Clinically, the simultaneous measurement of vitamin A and vitamin E is useful and important not only to clarify the relationship between these vitamins but also to evaluate the effect of supplementary therapy. HPLC has become a common method for measuring vitamins because of its high sensitivity and accuracy. Some methods for the simultaneous measurement of retinol and tocopherols using reversed-phase columns have been reported [1–4], but an improvement is achieved with the present method, namely the simultaneous separation of retinol and all four tocopherol homologues within 1 h. In addition, the peak resolution ($R_s$) of β- and γ-tocopherol was 1.26. The method satisfied the criterion of 4 σ separation [8]. The effective separation is mainly due to the column used. Another type of $C_{18}$ column was tested under the conditions in Table II, but it did not separate β- and γ-tocopherols. Although YMC-PACK A-302 S-5 120A ODS is a commercially available, common type of ODS column, the slurry packing technique differentiates it from other $C_{18}$ columns. Information about the slurry packing technique is not available from the manufacturer, however.

Using normal-phase columns all four tocopherols can be separated in a short time [7,16–19]. However, there have been no reports of the simultaneous determination of retinol and all four tocopherols using normal-phase columns, as far as we are aware. As Handelman *et al.* [20] reported, reversed-phase columns have some merits compared with normal-phase columns. When reversed-phase columns are used to determine tocopherols, δ-, γ-, β- and α-tocopherols are eluted in that order, whereas when normal-phase columns are used they are eluted in the opposite order. Because earlier peaks are sharper and higher than later peaks, the sensitivity for earlier peaks is higher than that for later peaks. Therefore, β-, γ- and δ-tocopherols can be determined with high-sensitivity using reversed-phase columns. In contrast, the sensitivity for α-tocopherol is low with reversed-phase columns. The sensitivity for α-tocopherol was 5 ng by the method of Russell *et al.* [1] using a reversed-phase column. Even compared with their data, the sensitivity for α-tocopherol was low with our method (10 ng). However, when our method was applied to rat liver, the α-tocopherol level was high enough to be determined.

The biopotency of α-tocopherol is considered to be the strongest among the four tocopherol homologues [21]. The concentration of α-tocopherol in animal tissues was shown to be higher than those of β-, γ- and δ-tocopherols in our previous studies. In contrast, γ-tocopherol is mainly found in the Japanese diet [22]. It is still unclear whether tocopherol homologues are metabolized in the same manner. There are some reports that the metabolism of γ-tocopherol is not the same as that of α-tocopherol [23–25]. This could imply that the effects of each tocopherol on retinol metabolism may be different. Our method can be used as a first step to clarify the relationship between retinol and individual tocopherol homologues.

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