

Determination of Serum Tocopherols by High Performance Liquid Chromatography

HITOSHI KATO, HIROSHI TANIMURA and YORINORI HIKASA

The 2nd Department of Surgery, Faculty of Medicine, Kyoto University
(Director: Prof. YORINORI HIKASA)

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Abstract

A simple and rapid method was desired to determine all the analogues of Vitamin E; α -, β -, γ - and δ -tocopherols in human serum with high performance liquid chromatography. Tocopherols, and tocol as an internal standard, were extracted into n-hexane and injected onto a Nucleosil-5NH₂ column with a mobile phase of 45/55 (by vol.) isopropyl ether/n-hexane. The eluted compounds were detected by a fluorescence spectrophotometer and calculated according to peak-height ratios to the internal standard. The time required was less than 10 minutes. The within-day precisions for all tocopherols were less than 2.1%, and the recoveries from fortified serum were from 94% to 100%. The average levels of α -, β -, γ -, δ - and total tocopherol in 19 sera of healthy Japanese were 9.1 ± 1.7 mg/L, 0.1 ± 0.1 mg/L, 0.8 ± 0.4 mg/L, 0.0 mg/L, and 10.2 ± 0.2 mg/L, respectively. This rapid method for complete separation of tocopherols was applied in various diseases to know any difference among them, and in surgical field to assess the influence of operation on serum tocopherol levels.

Introduction

Recent reports have pointed out the importance of Vitamin E as an antioxidant or membrane stabilizer^{7,15}. It is necessary to determine each analogue of Vitamin E separately, as their biological activities differ greatly².

Many methods of determining tocopherols have been described. The routine fluorometric methods were simple, but the individual tocopherols could not be differentiated^{12,13}. Thin-layer chromatography¹⁰ and combined thin-layer/gas liquid chromatography¹⁴ could separate them, but these methods are complex. Liquid-solid chromatography could separate them directly, but required considerable time¹⁷.

High performance liquid chromatography has been used recently, and a few good results have been obtained^{1,5,6,8}. However, more improvements were desired in respect to time required, separation of the analogues and reproducibility.

Key words: High performance liquid chromatography, Vitamin E, Intravenous fat emulsion.

索引語: 高度液体クロマトグラフィー, ビタミンE, 静注用脂肪乳剤.

Present address: The 2nd Department of Surgery, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto, 606, Japan.

The present paper describes a rapid, more sensitive and completely separating method using high performance liquid chromatography equipment with a spectrofluorophotometer for detecting, with good reproducibility, the analogue of Vitamin E in serum.

Materials and methods

Apparatus

A Hitachi 635 type high performance liquid chromatography and a Hitachi 204S fluorescence spectrophotometer (Hitachi, Tokyo, Japan) were used, also a 4 mm i.d. $\times$ 250 mm stainless-steel column packed with 5 μ m Nucleosil-5NH₂ (Macherey Nagel, West-Germany). The detector with a flow-through cell 2 mm in path width was monitored with 298 nm excitation and 325 nm emission. The peak heights and their ratios were calculated with a Digital integrator TR-2221 (Takeda Riken, Tokyo, Japan).

Reagents

The n-hexane used was a product prepared for pesticide residue analysis (Wako Pure Chemical, Osaka, Japan). Ethanol and isopropyl ether were guaranteed reagents obtained from Kanto Chemical, Tokyo, Japan. Isopropyl ether was washed with 10% NaOH to remove the antioxidation materials and then washed again with distilled water until the material was neutral. Pure α -, β -, γ - and δ -tocopherols, which were separately isolated from soy bean serum and purified by ion exchange chromatography, and tocol were donated by Eisai, Tokyo, Japan.

Method

0.5 ml of serum was added to 0.5 ml of distilled water in a 10 ml brown centrifuge tube with a glass-stopper and mixed well with a vortex mixer. Then 1.0 ml of ethanol containing 7 μ g of tocol as an internal standard was added. After stirring, 5 ml of n-hexane was added and mixed with the mixer (160 rpm, 1 min.), and 4 ml of the supernatant was dried under nitrogen gas flow at 40°C in a water bath. The residue was redissolved in 50 μ l of n-hexane and 20 μ l was injected onto the column. The chromatographic conditions are shown in Table 1.

To get the standard curves, samples of known volume of α -, β -, γ - and δ -tocopherols ranging from 0.005 μ g to 25 μ g (representing 0.03 mg/L to 156 mg/L) were injected onto the column with an equal volume of tocol. Curves were drawn by plotting the peak height ratios of tocopherols to tocol against their weight ratios.

Table 1. Chromatographic conditions.

Gel;	Nucleosil-5NH ₂ (5 μ)
Column;	4 i.d. $\times$ 250 mm
Column temperature;	Ambient
Mobile phase;	Isopropyl ether: n-Hexane (45 : 55)
Flow rate;	1.3 ml/min.
Pressure;	75 kg/cm ²
Detector;	Hitachi 204S fluorescence spectrophotometer (Ex. 298 nm, Em. 325 nm)

Results and discussion

The chromatogram obtained for standard solutions containing 5.95 μg of α -, 5.78 μg of β -, 5.50 μg of γ -, and 4.98 μg of δ -tocopherol and 6.73 μg of tocol is shown in Figure 1. Each tocopherol separated distinctly, and all the analogues were detected within 10 minutes. The detection limits were 0.06, 0.06, 0.07 and 0.07 mg/L for the four tocopherols respectively. Linear relationships between peak-height ratios and mass ratios were noted up to 60 mg/L. At higher concentrations, the slopes of the lines decreased. The regression lines were as follows; α -tocopherol $y=3.324x+0.095$, β -tocopherol $y=4.794x+0.027$, γ -tocopherol $y=4.794x+0.072$, δ -tocopherol $y=3.600x+0.027$.

The chromatogram of serum tocopherols of a healthy male is shown in Figure 2. The within-day precisions were 1.6%, 1.5% and 1.3%, for α -, β - and γ -tocopherol respectively (in 10 consecutive injections of normal serum, the mean and SD of the tocopherols were 9.7 ± 0.2 , 0.1 ± 0.0 , 1.0 ± 0.0 and 0.0 mg/L).

The day-to-day precisions were 3.3%, 4.4% and 4.6% (the same serum). Analytical recoveries for fortified serum were from 94 to 100% (Table 2).

Nineteen sera of healthy adults were analysed by this method (Table 3). α -Tocopherol, which has the highest biological activity, accounted for about 90% of the total tocopherol, β - for 1-2%, and γ - for 8%; δ -tocopherol was not detected. This proportion is consistent with that of former reports^{3,4)}.

The serum levels of α -tocopherol in various diseases are shown in Figure 3. Patients with

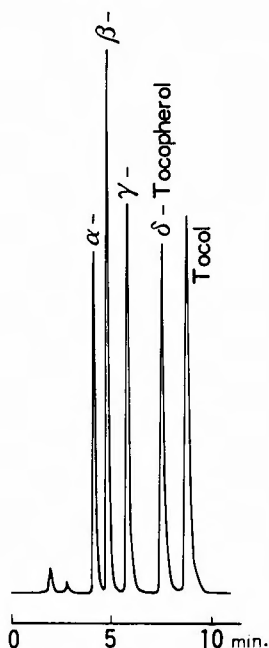


Fig. 1. Chromatogram of standard solution containing α -, β -, γ -, δ -tocopherols and tocol

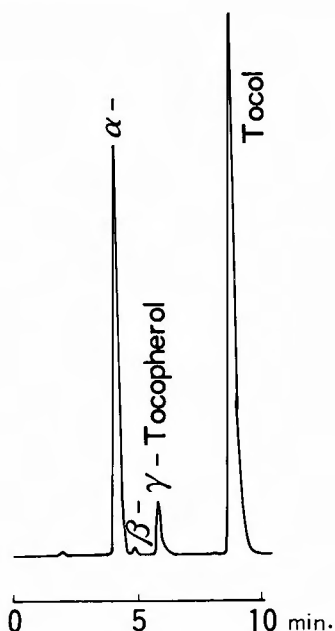


Fig. 2. Chromatogram of serum tocopherols in a healthy male

pancreatic diseases, such as pancreatic cancer and pancreatitis, had lower α -tocopherol (5.7 ± 1.2 mg/L) and total tocopherol (6.2 ± 1.3 mg/L) levels. In biliary atresia very low values were seen; α -tocopherol 2.3 ± 1.4 mg/L and total tocopherol 2.9 ± 0.6 mg/L. The patients with biliary atresia were infants, and it has been reported that new-born babies and infants have lower levels of

Table 2. Recovery of each tocopherol.

	Serum Toc. A (μ g)	Added Toc. B (μ g)	Total Toc. A + B (μ bg)	Observed Toc. C (μ g)	Recovery Toc. (C-A)/B (%)
α -Toc.	4.76	1.50	6.26	6.26	100
β -Toc.	0.10	1.24	1.34	1.26	94
γ -Toc.	1.14	1.36	2.50	2.44	96
δ -Toc.	0.00	1.04	1.04	1.00	96

Table 3. Serum tocopherols in normal subjects ($n=19$).

Tocopherol	Concentration (mg/L)	
	Range	Mean
α -Toc.	6.7-11.9	9.1
β -Toc.	0.1- 0.2	0.1
γ -Toc.	0.2- 1.3	0.8
δ -Toc.	0.0- 0.0	0.0
total	7.8-12.2	10.2

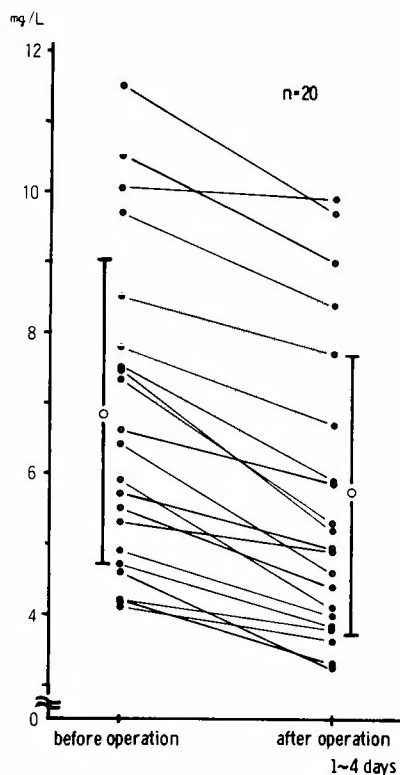


Fig. 4. Serum level of α -tocopherol before and after operation

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和文抄録

高速液体クロマトグラフィーによる血清
トコフェロール同族体の測定

京都大学医学部外科学教室第2講座 (主任: 日笠頼則教授)

加藤 仁司, 谷村 弘, 日笠 頼則

ヒト血清中ビタミンE同族体, 即ち α , β , γ , δ -トコフェロール測定の為, 簡便で迅速な測定方法が望まれていた. 今回, 我々はトコロールを内部標準として, ビタミンE同族体を n -ヘキサン中に抽出し, スクレオシルー 5NH_2 (5μ) カラムを使用し, イリプロピルエーテル・ n -ヘキサン (45:55) を移動相とする高速液体クロマトグラフィーを開発した. 検出は螢光光度計にて行ない, 内部標準との波高比にて濃度を計算した. 測定時間は10分以内で, 日内変動は2.1%以下, 添加回収率は94~100%であった. この測定方法を応用して, 1) 健常人19名における α , β , γ , δ 及び総トコフェロール値は, 平均 $9.1 \pm 1.7 \text{ mg/L}$, $0.1 \pm 0.1 \text{ mg/L}$, 0.8 ± 0.4

kg/L , 0.0 kg/L , $10.2 \pm 0.2 \text{ mg/L}$ であった. 2) 種々の疾患における血清中のトコフェロール値を測定した結果, 特に脾疾患, 胆道閉鎖症において α -トコフェロールが著明に減少していた. 3) 脂肪乳剤上には 20-30 mg/500 ml の総トコフェロールが含まれ, 60~70%は γ , 25~35%は δ で5%以下が α 及び β -トコフェロールであった, その投与により, 血清中の γ 及び δ -トコフェロールの上昇も認められた. 4) 手術侵襲による α -トコフェロールの減少は平均20%である. 等の結果が得られ, 臨床応用に十分役立つ測定法であるといえよう.