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Rapid determination of vitamins A and E in serum with surfactant as a diluent by column-switching high-performance liquid chromatography

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Abstract

The rapid and simple determination of fatty vitamins (vitamins A and E) in serum by high-performance liquid chromatography with a column-switching technique was investigated. The dilution of serum with an aqueous solution containing surfactant and organic solvent and the use of an aqueous solution with organic solvent as a sample pretreatment were an effective means of improving the sample recovery. To prevent sample decomposition during storage, the addition of an antioxidant reagent into the diluent was required. Under the optimal conditions, the relative standard deviation (R.S.D.) values against the standard samples were 1.12% (16.7 I.U./dl), 0.25% (333 I.U./dl) for vitamin A, and 1.02% (0.43 μg/ml), 0.45% (8.5 μg/ml) for vitamin E, respectively. The relative coefficients (r^2) between the sample amounts in serum and the peak areas were 1.0000 in the range from 16.7 to 667 I.U./dl (for vitamin A) and 0.9998 from 0.434 to 17.46 μg/ml (for vitamin E). The recoveries of vitamins from spiked serums were ca. 100% (vitamin A) and ca. 86% (vitamin E), respectively. By the combination of an on-line deproteination column and diluting solution, the simple and rapid determination of fatty vitamins could be routinely achieved in 18-min intervals. © 1998 Elsevier Science B.V.

Keywords: Column-switching; Vitamins

1. Introduction

Fatty vitamins such as retinols [vitamin A (VA) and its analogues] and tocopherols [vitamin E (VE) and its analogues] play various important roles in the growth, metabolism and antioxidation of living things. Recently, such fatty vitamins are attracting interest not only as a nutrient in food chemistry but also as a preventive of cancer, aging and other diseases in medicine.

High-performance liquid chromatography (HPLC) is one of the most powerful tools for determining fat soluble vitamins. Both normal-phase liquid chromatography (NP-LC) [1–3] and reversed-phase liquid chromatography (RP-LC) [4–6] have been widely utilized for separating fatty vitamins. In both chromatographic methods, VA and VE have to be extracted from sample solutions such as serum, plasma and internal organs, with organic solvents and followed by centrifuging and concentrating prior to apply to the HPLC system. These processes are time-consuming, troublesome and can give rise to serious human error.

There are two pretreatment types (as reviewed by Wyss [7]), in the application of the column-switching technique combined with HPLC for analyzing drugs

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in biological fluids such as serum, plasma, urine and homogenized organs, (1) solid-phase extraction (SPE) and (2) precolumn use. In the latter method, Yoshida and coworkers [8–10] reported a simple sample pretreatment process with column-switching HPLC for analyses of drugs in human plasma. They employed octadecylsilyl bonded silica (ODS) coated with bovine serum albumin (BSA). Pinkerton and coworkers [11–13] have also suggested use of similar double functional gels which were produced with hydrophobic peptides and enzymes. Recently SPE has been used for the on-line determination of retinol and relatives [14]. Low-molecular-mass drugs in the serum sample can be analyzed by the on-line column-switching HPLC technique by using these pretreatment tools.

Wyss and Bucheli [5] reported the application of retinoids in plasma using a column-switching HPLC technique. In their process, there are several problems; (1) use of an alkaline solution as a diluent of plasma, (2) long analytical time, (3) use of an ODS gel for the deproteination process and (4) less attention paid to the effect of oxidation of samples. Silica based packing materials are a disadvantage under such alkaline solutions. Bare ODS columns adsorb serum proteins resulting in deteriorating column performance over the course of 40–50 injections. In addition, VA and VE are essentially sensitive towards the oxidation reaction caused by oxygen in air or in an eluent, UV light and oxidizing agents such as transition metals (mainly iron, copper in a column and/or sample solution). It is important to pay more attention to sample decomposition by oxidation.

We report the investigation of the simple, rapid and simultaneous determination of VA and VE in serum by combining column-switching HPLC with an unique sample pretreatment column.

2. Experimental

2.1. Chemicals

The standard retinol (VA, purity;99%) and DL-α-tocopherol (VE, purity; 96%) were purchased from Fulka (Tokyo, Japan) and Kishida (Osaka, Japan), respectively. Brij 35, Triton X-100, sodium dodecyl sulfate (SDS) from Kishida were of analytical-grade. Analytical-grade antioxidant reagents, ascorbic acid, pyrogallol and ethylenediaminetetraacetic acid 4 sodium salt (EDTA·4Na) were from Kishida. HPLC-grade acetonitrile and ethanol, analytical-grade hexane and isopropanol were from Wako (Tokyo, Japan). Distilled water was prepared using a Milli-Q system (Millipore, Tokyo, Japan).

2.2. Instrumentation

The apparatus employed consisted of a SC-8020 system controller (SC), a CCPM II dual pump (P1 for pretreatment and washing eluents, P2 for analytical eluents), a CO-8020 column oven (CO) at 40°C, a VC-8020 valve controller (VC) with MV-8020 motor valves (M1), a AS-8020 autosampler (AS) and a fluorescence detector (FS) at excitation 340 nm (for VA), 298 nm (for VE) and emission 460 nm (for VA), 325 nm (for VE), respectively. The wavelength of the FS was automatically switched at 11 min after injection. The line filter (F, 1 μm) was positioned between the autosampler and M1, P1 and M1, respectively. The schematic flow diagram is illustrated in Fig. 1. (A) and (B) in the figure show the sample pretreatment and analytical status.

A TSKgel ODS-80Ts [for analytical column (AC), 5 μm, 150 mm×4.6 mm I.D.], a TSK guardcolumn ODS-80Ts [for guard column (GC), 5 μm, 15 mm×3.2 mm I.D.] and a TSK precolumn BSA-80Ts [for

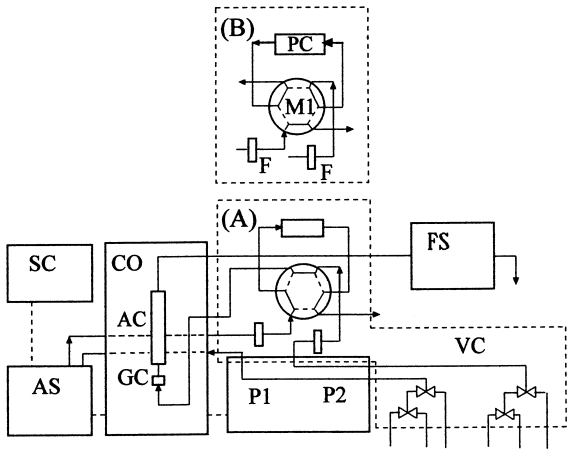

Fig. 1. Schematic flow diagram of HPLC system. The details are shown in Section 2.2.

pretreatment column (PC), 13 μm, 10 mm×4.6 mm I.D.] were obtained from Tosoh (Tokyo, Japan).

2.3. Sample preparation

2.3.1. Standard vitamin solutions

6.31 g of pyrogallol solved in 500-ml ethanol was used as a diluting solution (D1) for standard sample solutions. The concentrated VA (A1) and VE solutions (E1) were separately prepared by solving each 10 mg of VA and VE in 250 ml and 100 ml of D1, respectively. These solutions were stored in brown bottles at −20°C.

The original standard vitamin mixture solution (M1) was made by mixing each 5-ml portions of A1 and E1 and then diluting with D1 in a 50-ml brown bottle. The diluted standard solutions (M2–M6) were prepared by diluting 5-, 10-, 25-, 50- and 100-times with D1, respectively.

The diluent of standard and serum samples was prepared by mixing 70 ml of 200 m*M* SDS solution and 30 ml of ethanol (D2). Other diluents containing EDTA, pyrogallol or ascorbic acid were also made using the protocol described above.

Standard solutions for calibration curves were prepared by mixing 100-μl portions of the vitamin standard solutions (M2–M6) and 900-μl portions of D2 in a siliconized sample cup with a Vortex mixer.

2.3.2. Spiked and serum samples

In the recovery study, the spiked solutions were made by mixing 10-μl portions of M2–M6, 90-μl portions of fresh human serum and 900-μl portions of D1 in brown bottles. The standard solutions were prepared in the same manner except using ethanol instead of human serum. For the serum blank sample, 10 μl of ethanol, 90 μl of human serum and 900 μl of D1 were mixed in the brown bottle. These diluted solutions were filtered with a 0.45-mm cellulose disk filter in advance. The sample solutions were cooled at 15°C in the autosampler.

2.4. Chromatographic procedure

Vitamins were stepwisely eluted by 80% ethanol for VA (S2) and 87% ethanol aqueous solution for VE (S3). An aqueous 20% ethanol solution with P1 was used as a pretreatment solution. Flow-rates of the eluent and pretreatment solution (S1) were 1.0 ml/min and 1.5 ml/min, respectively. The pre-column (PC) was washed with S3 for 3 min.

The injection volume was 300 μl portions of diluted standard, spiked and human serum samples by the autosampler.

The column-switching valve (M1) was switched to (B) status at 3 min and back to (A) at 4 min after injection. The sample trapped on the PC was eluted out to an analytical column (AC) with the backflush flow. The wavelength in the FS was changed at 2 (for VA) and 11 min (for VE) after injection. Eluents S2 and S3 were stepwisely switched to S3 at 2 min and to S2 at 11.5 min after injection.

Continuous analysis with the autosampler was carried out over an 18-min interval.

For the extraction sample, the analytical ODS column (TSKgel ODS-80Ts, 15 cm×4.6 mm I.D.) was employed. Twenty μl portions of the concentrated sample solutions were directly injected onto the ODS column. Other conditions were the same in the on-line procedure.

2.5. Extraction procedure

Two ml of human serum was treated with 2 ml of ethanol and 10 ml of hexane with mixing for 30 s and followed by centrifuging at 3000 rpm for 5 min. The hexane supernatant was stored at 15°C. Ten ml of hexane was added to the residue with mixing for 30 s and centrifuged at 3000 rpm for 5 min. After mixing both hexane supernatants, hexane was evaporated with a N_2 stream. Two-hundred μl of isopropanol was added to the residue. A 20-μl portion of the isopropanol solution was injected.

For the recovery study, 100-μl portions of M2–M6 were added to 1900-μl portions of human serum and deionized water as spiked and standard samples. For the serum blank sample, 100 μl of ethanol was added to 1900 μl of human serum. These solutions were also treated in the same manner as described above.

2.6. Determination of sample recoveries

The recoveries of vitamins in the on-line and the extraction process were calculated by the following equation,

Table 1
Effect of surfactant in diluent on vitamin recoveries

Surfactant	Recovery (%)			
	Vitamin A		Vitamin E	
	Standard	Serum	Standard	Serum
30% Ethanol	89.9	77.1	27.8	14.8
Triton X-100[a]	104.3	95.2	102.6	15.9
Brij 35[a]	96.6	90.9	87.9	38.1
SDS[a]	107.4	98.9	103.5	86.5

Chromatographic conditions were the same as in Fig. 3, except using above surfactant solutions instead of SDS.
[a] Contained 30% ethanol.

$$\text{Recovery}\,(\%) = 100(A_{SS} - A_B)/A_S$$

where A_{SS}, A_B and A_S are the peak areas of VA and VE from the spiked serum sample, blank serum and standard sample, respectively. The recoveries presented are the averages of several measurements ($n=3$ or 6).

3. Results and discussion

3.1. Effect of organic solvent in diluent on recovery

In general, dilution of serum samples with an aqueous solution is an effective means to lower the viscosity of sample solutions and to prevent protein precipitation. In the case of diluting the vitamin standard solution with water, no recoveries of VA and VE were observed as they existed as oily drops in the eluent when passing through the pretreatment column. The addition of a diluent containing alcohol (over 80%) was required to improve recoveries of vitamins. However, a diluent containing high concentrations of alcohol caused formation of an insoluble precipitate in the serum samples. It is well known that fatty soluble vitamins, especially VA, are strongly bound to retinol-binding protein (RBP) in blood [15]. Small amounts of alcohol in the diluent (ca. below 30%, v/v) were not adequate to eliminate VA and VE from RBP.

3.2. Effect of surfactant in diluent on recovery

The effect of surfactant, which has been utilized as a solubilizing reagent against proteins, on recovery was examined for the purpose of eliminating VA from RBP and solubilizing fatty vitamins into the eluent. Table 1, shows that the recoveries of the standard vitamins were good with the use of each surfactant solution. However, lower recoveries were obtained for spiked serum samples by using neutral surfactants, Triton X-100 and Brij 35. Alkyl sulfates gave excellent results, especially in the use of SDS. In the range of SDS concentration from 0 *M* to 300 m*M*, the appropriate concentration of SDS in a diluent was over 200 m*M*. It seems that the SDS micelles act as an extracting and eliminating agents against fatty soluble VA and VE. The addition of SDS did not affect practically on the retention times and column stability.

3.3. Comparison of recovery on on-line and extraction procedure

The recovery of vitamins with the on-line HPLC and solvent extraction procedure is summarized in Table 2. Although the recoveries of VA and VE were

Table 2
Comparison of recovery of vitamins with solvent extraction and the on-line HPLC method

	Recovery (%)	R.S.D. (%, $n=3$)
Solvent extraction		
Vitamin A (67.9 I.U./dl)	101.9	12.62
Vitamin E (0.39 μg/ml)	118.5	3.21
On-line HPLC		
Vitamin A (74.0 I.U./dl)	102.8	2.48
Vitamin E (0.51 μg/ml)	85.9	1.44

Chromatographic conditions were as described in Section 2.4.

almost quantitative in both methods, the relative standard deviation (R.S.D.) values of the extraction procedure were larger than those of the on-line HPLC method.

3.4. Sample stability in diluent solution

The decrease of VE recovery with the time-course was observed in the continuous determination as shown in Fig. 2A. This phenomenon would imply that an oxidation reaction is taking place in the diluted solution. The effect of adding an antioxidant to the diluent was investigated to prevent the reduction of peak areas.

The stability of VE could be improved by using pyrogallol and EDTA. However, using EDTA seems to be recommended because the recovery of VE was higher than that in the pyrogallol use. In EDTA use, the optimal pH range must be around 8 (adjusted

Table 3
Repeatability of vitamin determination in human serum by the on-line HPLC method

	Repeatability (R.S.D., %, $n=6$)
Standard sample in spiked serum	
Vitamin A	
(16.7 I.U./dl)	1.25
(33.3 I.U./dl)	1.28
(66.7 I.U./dl)	0.51
(333 I.U./dl)	0.26
Vitamin E	
(0.434 μm/dl)	1.12
(0.868 μm/dl)	0.85
(1.736 μm/dl)	0.67
(8.68 μm/dl)	0.35
Human serum	
Vitamin A	0.98
Vitamin E	0.65

Chromatographic conditions were the same as in Fig. 3.

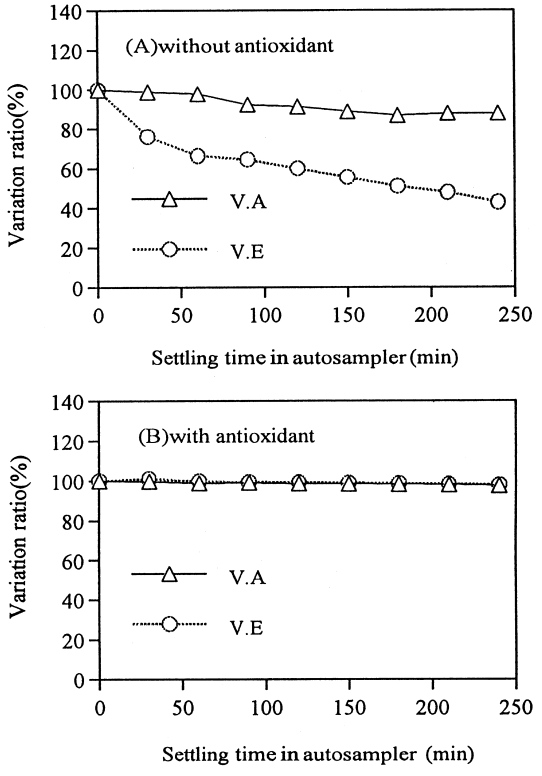

Fig. 2. Time-course of the variation ratio of VA and VE in diluted solution without (A)/with (B) antioxidant. EDTA was employed as an antioxidant. Other conditions as in Fig. 3.

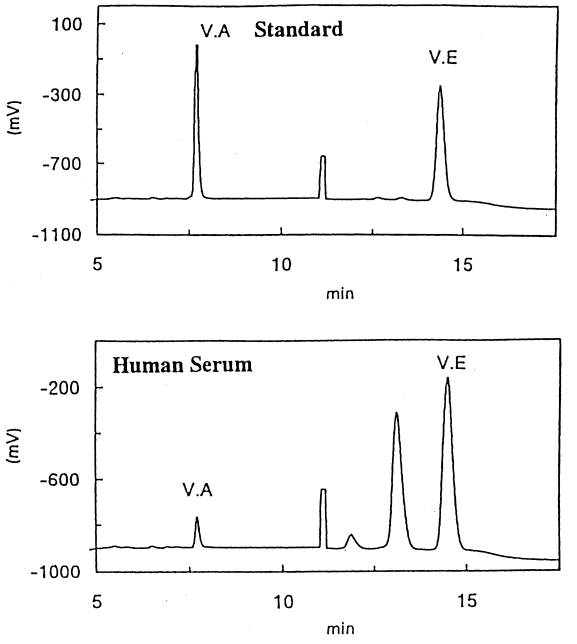

Fig. 3. Chromatogram of VA and VE in standard and human serum samples by column switching HPLC. Conditions: eluent: 80% ethanol (for VA), 87% ethanol (for VE), washing solvent: ethanol, diluent: 200 m*M* SDS–ethanol (70:30) containing 200 m*M* EDTA and 0.3% H_3PO_4, injection volume 300 μl (10-fold dilution). Sample: standard sample (A), human serum (B). Other conditions as described in Section 2.4.

with phosphoric acid). As illustrated in Fig. 2B, both recoveries were almost constant during storage in the autosampler.

Under the standard conditions, the repeatability with the standard and serum samples is summarized in Table 3. The R.S.D. values at high and low concentrations of the standard vitamins and spiked serum samples were adequately acceptable (under 1.5%). The relative coefficients (r^2) in the calibration curves were 1.0000 (16.7–667 I.U./dl for VA) and 0.9998 (0.434–17.36 μg/ml for VE) and the detection limits (S/N ratio = 3) were 1.67 I.U./dl (for VA) and 0.05 μg/ml (for VE), respectively. The international unit (1 I.U.) for VA corresponds to 0.333 μg of all-*trans*-retinol.

Fig. 3 illustrates the chromatograms of VA and VE in the standard and human serum samples by using the on-line HPLC method. The amounts of vitamins in human serum were 185 I.U./dl for VA and 8.35 μg/ml for VE, respectively. VE isomers, δ- and β-tocopherol, were recognized in the front of the VE peak on the chromatogram.

4. Conclusions

The on-line determination of fatty soluble vitamins in human serum by using a column-switching HPLC technique was investigated. The BSA-80Ts pre-column enabled the direct and rapid deproteination from the serum samples. The use of the diluent containing the surfactant and organic solvent allows the excellent recoveries of VA and VE from the serum samples. To maintain the sample stability in the diluted solution, the addition of the antioxidant reagent into the diluent was required.

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