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TWO-DIMENSIONAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM FOR THE DIRECT DETERMINATION OF TOCOPHEROL DERIVATIVES IN PLASMA

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SUMMARY

A procedure for the quantitative extraction of tocopherol derivatives from plasma was systematically developed using an efficient liquid–liquid distribution process. An aqueous stationary phase was coated on silica gel or diatomaceous earth powder in a closed-bed glass column prepared by slurry packing. A liquid–liquid chromatographic system provided with a detector was introduced as a preliminary extraction process. Liquid–solid chromatography followed as the second dimension of the separation and was used to determine α-tocopherol nicotinate in the extract. Stationary and mobile phases in the two-dimensional liquid chromatographic system were optimized to determine tocopherol derivatives. A highly sensitive quantitation method for α-tocopherol nicotinate in plasma was developed by using liquid–liquid and liquid–solid chromatography in conjunction with column switching by a tube-type micro-evaporator.

INTRODUCTION

The increasing importance of monitoring drugs and their metabolites in plasma has prompted various liquid chromatographic studies directed toward the development of high-performance and highly sensitive analytical systems. Plasma is a complex matrix consisting of thousands of constituents, and the concentrations of target compounds present in it are usually low, thus making their determination difficult. It is for this reason that a selective fractionation procedure for enrichment of such compounds should be developed.

This paper described the design of a high-performance liquid–liquid distribu-

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tion system to improve the clean-up process. Extraction solvents were tested for quantitative sample recovery. To prevent contamination, the solvent composition was adjusted in the liquid–liquid distribution system using packed columns. Stepwise elution was conducted to achieve highly selective fractionation. To obtain high resolution in the separation of extracted components following fractionation, high-performance liquid chromatography (HPLC) was carried out as the second dimension of the system. Phase systems, including stationary and mobile phases in HPLC, were precisely optimized. The solvent composition in the liquid–solid chromatography was controlled according to a mathematical correlation between retention indices and binary solvent concentrations.

This paper describes a study of five tocopherol derivatives. The biological function of α-tocopherol (α-T) as a lipid antioxidant is well known. Various derivatives of tocopherol are currently used as drugs. α-Tocopherol nicotinate (α-TN) is a peripheral vasodilator used to improve blood circulation following coronary insufficiency and cerebral apoplexia [1]. It is widely used in Japan for the treatment of hypertension and arteriosclerosis. Thus, a practical procedure for the quantification of α-TN and its desmethyl homologue, γ-tocopherol nicotinate (γ-TN), in plasma was developed using two-dimensional separation.

EXPERIMENTAL

Materials

Five tocopherol derivatives, α-and γ-tocopherol (α-T and γ-T), α-tocopherol acetate (α-TA) and α- and γ-tocopherol nicotinate (α-TN and γ-TN), were of reagent grade and obtained from Nisshin Chemical (Tokyo, Japan). The purity of these samples was confirmed by silica gel thin-layer chromatography using various mobile phase systems, and 100 mg of each sample were dissolved in 100 ml of *n*-hexane. The standard solutions were stable at 4 °C for at least one month when protected from light. A working solution was prepared by diluting the stock solution 100-fold with *n*-hexane to a concentration of 10 μg/ml of each sample. The solvents, obtained from Wako (Osaka, Japan), were of reagent grade.

Liquid–liquid distribution ratio measurements

A 1-mg amount each of α-TN and γ-TN was added to 20 ml of distilled water. The insoluble portion was removed by filtration, and 5 ml of *n*-hexane and the same volume of each aqueous sample solution were equilibrated for one day. An aliquot of each two-layer solution was subjected to HPLC analysis. The distribution ratios were calculated by dividing the concentration of the upper organic layer by that of the lower aqueous layer. The ratios of α-and γ-TN were 1.04 and 1.52, respectively. When 2 ml of water were added to a two-layer phase consisting of 8 ml of *n*-hexane stock solution and 2 ml of methanol, the distribution ratios obtained were 85 for α-TN and 416 for γ-TN.

Batch extraction procedure for spiked plasma samples

A 20-μl volume of the working solution, containing 200 ng of each sample, was evaporated in a test-tube. Then 1 ml of beagle dog plasma was added and vortex-

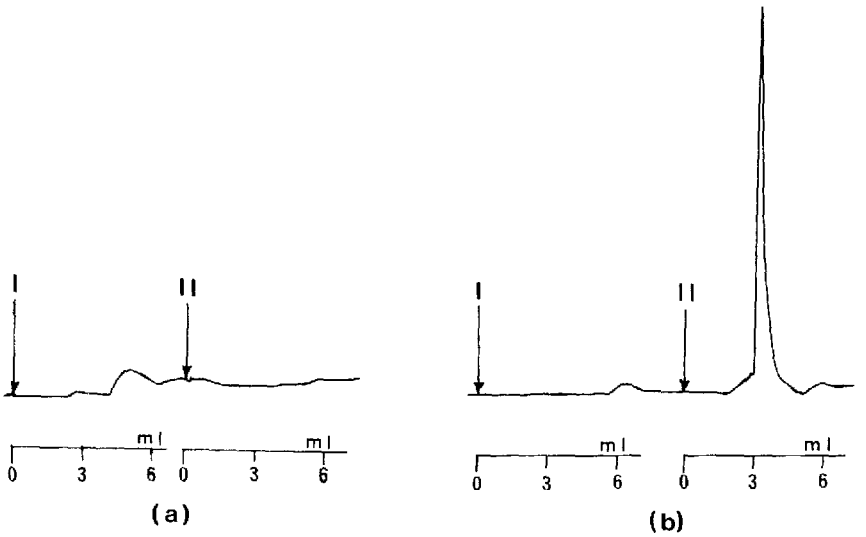

Fig. 1. Chromatograms of tocopherol derivatives extracted from plasma by an LL column. Mobile phase, *n*-hexane (I) and methanol-saturated *n*-hexane (6%, v/v) (II); flow-rate, 0.5 ml/min; wavelength, 264 nm; range, 0.32 a.u.f.s. (a) Blank plasma; (b) plasma containing tocopherol derivatives.

mixed for 1 min. The spiked plasma was extracted with 20 ml of *n*-hexane or *n*-hexane–methanol (4:1, v/v) in a separation funnel. The organic layer was separated and evaporated to dryness. The residue was dissolved in 50 μl of a mobile phase solvent, and 10 μl of this solution were applied to the HPLC column.

Liquid–liquid distribution process using columns

A closed-bed column for liquid–liquid distribution (LL column, 20 cm×4 mm I.D.) was prepared with a glass tube and a PTFE fitting (CIG column system, Kusano Scientific, Tokyo, Japan) and packed with silica gel, 70–230 mesh particle size, 60 Å pore size (Kieselgel 60, Merck, Darmstadt, F.R.G.). A slurry-packing procedure was used with an HPLC pump. The number of theoretical plates (N) and dead volume (V_0), calculated using benzene and ethyl phthalate as samples and *n*-hexane–ethyl acetate (9 : 1, v/v) as the eluent were $N=175$ and $V_0=2.48$ ml for benzene and $N=83$, V_R (retention volume) $=8.98$ ml for ethyl phthalate.

The column outlet was connected to the UV detector (Uvidec 100V, Jasco, Tokyo, Japan) to monitor the peaks. For determination of α-TN and γ-TN, the detector was set at 264 nm and the detector range was 0.32 or 0.16 a.u.f.s. A Trirotar-V pumping system (Jasco, Tokyo, Japan) and a Rheodyne 7125 sample injector were used. *n*-Hexane was pumped into the column at a flow-rate of 0.5 ml/min, followed by injection of 0.2 ml of beagle dog plasma spiked with tocopherol derivatives (Fig. 1a). α-TN and γ-TN were eluted with methanol-saturated *n*-hexane (6%, v/v) (Fig. 1b). After peak fractionation and evaporation to dryness, the residue was dissolved in a mobile phase solvent for the HPLC determination.

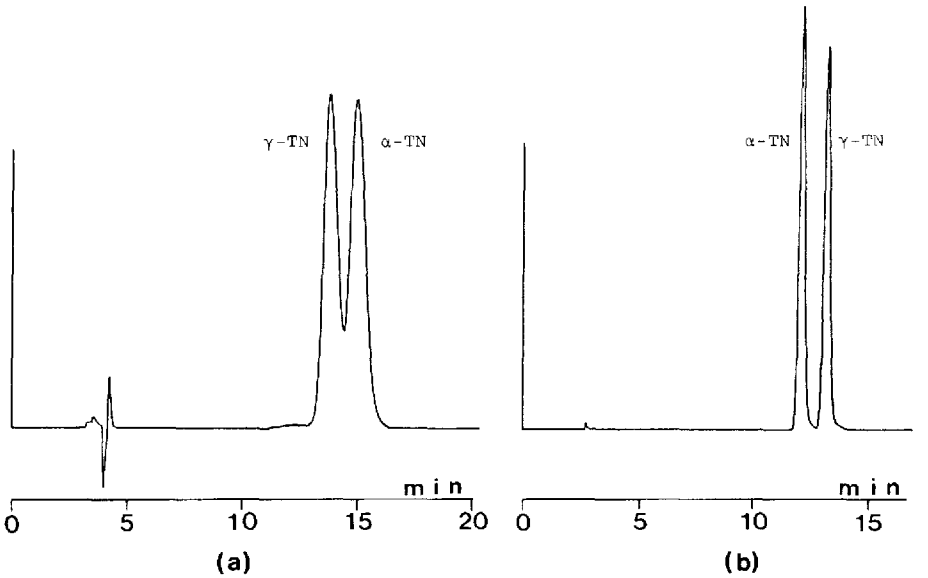

Fig. 2. Comparison of reversed-phase and normal-phase chromatograms. (a) Mobile phase, methanol; column, Shodex F-511B, 5 μm, 25 cm×4 mm I.D.; flow-rate, 1 ml/min; wavelength, 264 nm; range, 0.32 a.u.f.s; (b) mobile phase, 0.5% (v/v) ethanol–*n*-hexane; column, LiChrosorb Si 60, 5 μm, 25 cm×4 mm I.D.; flow-rate, 1 ml/min; wavelength, 264 nm; range, 0.32 a.u.f.s. Peaks: α-TN = α-tocopherol nicotinate; γ-TN = γ-tocopherol nicotinate.

A closed-bed diatomaceous earth column [2] was similarly prepared. It was packed with Celite powder, the average particle size of which was 100 μm (No. 545, Johns-Manville, Denver, CO, U.S.A.). The number of theoretical plates (N) and the dead volume (V_0) were $N=63$ and $V_0=1.63$ ml for benzene.

HPLC procedures for tocopherol derivatives

Pumping, injection and detection equipment for HPLC were the same as described in the section on liquid–liquid distribution. A silica gel column (LS column), LiChrosorb Si 60 (25 cm×4.0 mm I.D., Cica-Merck, Tokyo, Japan) was used as the normal-phase HPLC system. Eluents were binary solvents consisting of *n*-hexane and a polar solvent such as ethanol, *n*-propanol, diethyl ether or diisopropyl ether. An ODS column, Shodex F-511B (25 cm×4 mm I.D., Showa Denko, Tokyo, Japan) was used for reversed-phase chromatography. The eluent was methanol, methanol–water or acetonitrile–water. The flow-rate was 1 ml/min. The UV detector was set at 264 nm.

Retention indices were calculated as follows: $k' = (V_R - V_0) \,/\, V_0$, where k' is the capacity ratio. The dead volume was determined using benzene as the substance least retained in normal-and reversed-phase systems. The column efficiency, expressed as the number of theoretical plates, was measured for α-TN as follows : silica gel column, ethanol–*n*-hexane (0.5%, v/v), $k'=3.44$, $N=6572$ and asymmetry factor (As) = 1.01; ODS column, methanol, $k'=3.57$, $N=1920$ and $As=1.16$ (Fig. 2a and b).

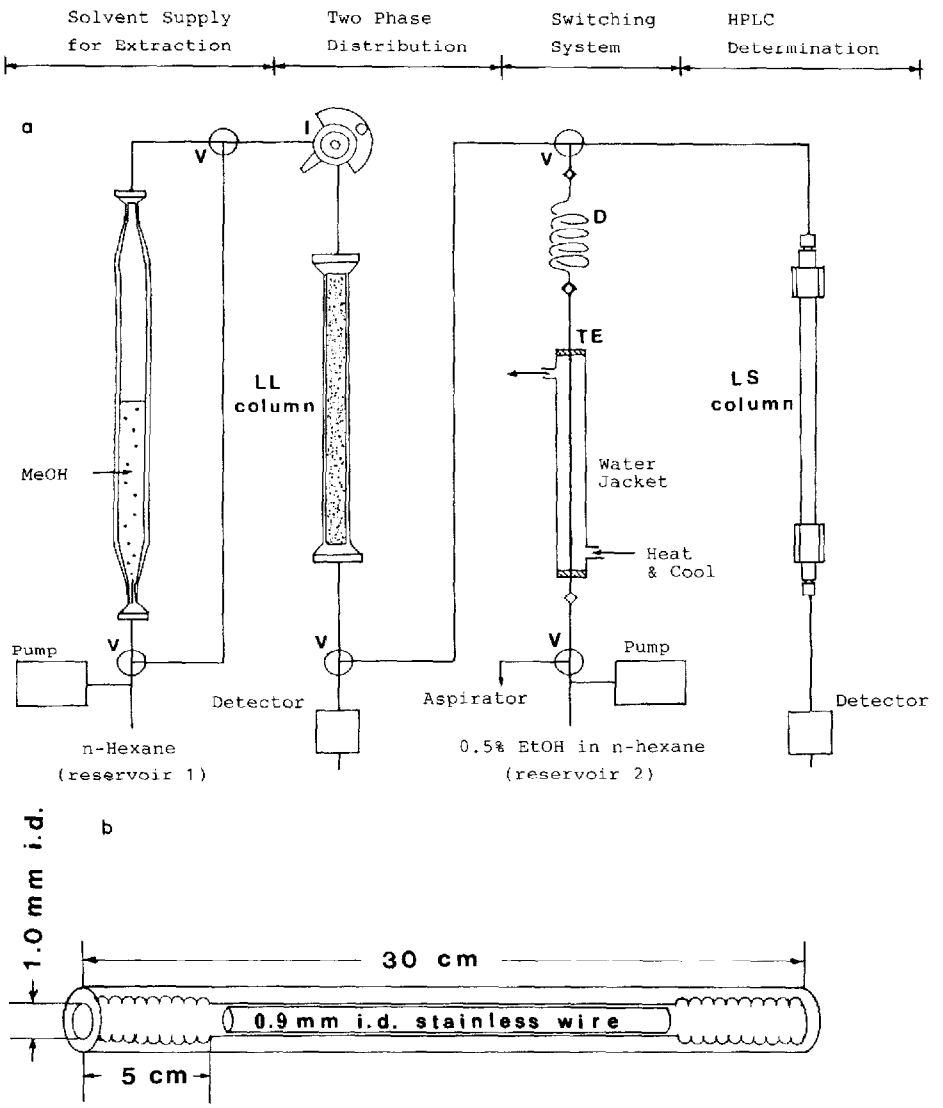

Fig. 3(a). Diagram of the two-dimensional liquid chromatographic system equipped with a tube evaporator. I = injector; LL column = liquid-liquid distribution column for extraction; LS column = liquid-solid analytical HPLC column; D = damper; TE = tube evaporator; V = three-channel valves. (b) Detailed view of the tube evaporator. The inside surface of both the inlet and outlet of the PTFE tube was scratched with a sharp stainless-steel wire (0.5 mm diameter), to increase its area. To increase the heat conductivity and prevent solute diffusion, a stainless-steel wire (0.9 mm diameter) was inserted into the PTFE tube (1.0 mm I.D.).

Column-switching system

On-line extraction equipment for the α-TN determination is shown in Fig. 3a. The inlet of the silica gel column (LL column) for liquid–liquid distribution as described above was connected via two mechanical valves to the pump supplying the *n*-hexane. The sample plasma (0.2 ml) was first injected through the injection

port into the column filled with *n*-hexane, and *n*-hexane was then added at a flow-rate of 0.5 ml/min. The methanol–*n*-hexane mixture was prepared by passing *n*-hexane through the methanol phase in the glass tube situated between the LL column-inlet and pump. The droplet current of *n*-hexane saturated with methanol (ca. 6%, v/v) was introduced at the same flow-rate into the LL column.

The eluent was introduced into the tube evaporator via a mechanical valve in accordance with a timetable programmed in advance on the basis of preliminary experiments. The evaporator was made of PTFE tubing and stainless-steel wire which was placed inside the PTFE tube to decrease the dead volume [3,4] (Fig. 3b). A vacuum line was connected to the tube in which the sample fraction was to be subsequently evaporated to dryness under vacuum (water aspirator). The evaporator tube was heated at 50–60°C by a water jacket containing hot water, then cooled by the passage of cold water. The eluent, consisting of *n*-hexane and 0.5% (v/v) ethanol, was introduced into the tube evaporator from reservoir 2 by a valve to dissolve the residue.

The resulting solution was applied to the silica gel column (LS column) for HPLC determination. The flow-rate of the eluent was 1 ml/min. Baseline stabilization was necessary to ensure high sensitivity in a detection range of 0.0025–0.005 a.u.f.s. and recorder range of 2 mV. Calibration curves in the plasma assay were made on the basis of the peak-height ratios of α-TN plotted against γ-TN.

Determination of α-TN in plasma samples.

α-TN (100 mg) diluted with lactose was placed in a gelatin capsule and administered with 20 ml of water either after the beagle dogs had fasted overnight or after they had been fed 125 g of DS diet obtained from Oriental East (Tokyo, Japan). Four male beagle dogs weighed from 11.0 to 13.1 kg. Sixteen male human subjects were each given orally a 200-mg capsule of α-TN after a standard breakfast. Blood samples were obtained every 2 h and, to ensure accuracy of subsequent measurements, the interior of each syringe was washed with heparin solution prior to each sampling. The plasma samples obtained after centrifugation at 1000 *g* were frozen at −20°C until analysis.

RESULTS AND DISCUSSION

Liquid–liquid distribution process

The organic solvent extraction of solutes from aqueous plasma is widely used in drug monitoring as a typical pretreatment. However, it is not possible by such a batch process to collect target components quantitatively and to exclude contaminants that interfere with the quantitation.

For the extraction of tocopherol derivatives from plasma, *n*-hexane–methanol was selected in the present research [5]. When pure *n*-hexane was used, the distribution of the tocopherol derivative in the organic phase was very low. The distribution ratio of α-TN and γ-TN in *n*-hexane–water containing methanol was several hundred times higher, indicating that the quantitative recovery of sample

compounds was possible. However, the compounds recovered from spiked plasma were highly contaminated, resulting in lower resolution.

To avoid this, a packed column that would afford highly efficient liquid–liquid distribution via a multiple equilibrating process was used. The aqueous liquid–liquid chromatographic system included a slurry-packed closed-bed column, carrier pumping equipment and UV detector [2,6].

When *n*-hexane was used as the carrier solvent with an aqueous stationary phase and the silica gel column, no elution peak was obtained for the tocopherol derivatives. However, a sharp front peak appeared, which indicated a capacity ratio of 0–1, after several percent of methanol were added to the *n*-hexane. This phase system afforded complete fractionation of the solutes.

To demonstrate the applicability of this fractionation procedure to plasma, a plasma sample containing tocopherol derivatives was injected into the silica gel column filled with *n*-hexane. *n*-Hexane was continuously pumped through the column for several minutes. The expectation was that the organic solvent saturated with water would eliminate the lipophilic substances in the plasma, which interfere with resolution of the tocopherol derivatives. It was possible to extract a large amount of such compounds, but the peak height was not remarkable owing to the lower detection sensitivity (Fig. 1).

Since the concentration of methanol in *n*-hexane at saturation was 6%, a module for the dynamic saturation of *n*-hexane by droplet methanol flow was constructed (Fig. 3a). As a result of introducing methanol-saturated *n*-hexane into the column, a sharp elution peak representing tocopherol derivatives appeared, as shown in Fig. 1b. The fraction containing α-TN and γ-TN was collected and injected into the HPLC column. There was no interference with the resolution of tocopherol derivatives in this fraction. It is thus evident that the first elution with *n*-hexane prior to extraction of the tocopherol derivatives results in a successful clean-up of the extracts. The recovery of tocopherol derivatives added to the plasma sample was then determined, and the results indicated that the extraction was quantitative.

The silica gel column quickly deteriorated following injection of the plasma sample, possibly as a result of irreversible adsorption of highly polar macromolecular proteins in the plasma samples. Consequently, the column had to be washed with methanol and water prior to use. To prolong column life, a back-flush solvent should be allowed to flow through the column soon after fractionation.

Diatomaceous earth granules are a good support material for aqueous liquid–liquid distribution processes. A closed-bed diatomaceous earth column was thus prepared by the slurry-packing procedure [2]. Following slight modification of the eluent volume by adjustment of the phase ratio and column dead volume, the retention of tocopherol derivatives was essentially the same and the recovery was almost quantitative.

Liquid–solid chromatography separation

For the separation of tocopherol derivatives, a reversed-phase system consisting of an ODS column filled with aqueous methanol is commonly used [7–9]. However, with methanol, methanol–water or acetonitrile–water as the eluent, the

resolution of tocopherol and its homologues was poor (Fig. 2a). Thus, instead of a reversed-phase column, a normal-phase system consisting of a silica gel column and a non-aqueous solvent was examined. The result was superior column efficiency and good selectivity for tocopherol derivatives. Binary systems consisting of *n*-hexane and various solvents of greater polarity were examined. Minimal peak tailing and high resolution were found with *n*-hexane–ethanol. A chromatogram obtained with such a system is shown in Fig. 2b.

For optimization of the mobile phase , the correlation between the solute retention and the solvent composition was examined. A linear relation between the logarithms of the capacity ratio and the logarithms of polar solvent fractions was confirmed [10] and was completely consistent with our experimental results. Retention values obtained with the *n*-hexane–ethanol system are shown in Fig. 4.

From these values, the adsorption mechanism of tocopherol derivatives appears to be as follows: (1) α-TA, produced by acetylation of the hydroxyl group in α-T, has the lowest retention, so the hydroxyl group should contribute most to the adsorptivity of α-T; (2) the α-T and γ-T homologues were found to differ considerably in the degree to which they were adsorbed, indicating a significant steric effect in the adsorption of α-T due to the methyl group that is not present in γ-T; (3) the adsorption of nicotinates increased greatly and the mutual adsorption differences of α and γ homologues from free tocopherol derivatives decreased. The nicotinic acid portion of α-TN and γ-TN may thus possibly be responsible for the adsorption phenomenon, and the nitrogen atom in the pyridine ring may function as a stronger active site in these solutes than the hydroxyl group in free tocopherol molecules.

Determination of α-TN in plasma

On the basis of the liquid–liquid distribution and liquid–solid chromatographic retention behaviour described above, a method for the quantitation of α-TN in plasma was developed. γ-TN behaved similarly to α-TN during the solvent extraction and chromatographic retention studies, and thus was used as the internal standard for the quantitative determination of α-TN.

The extraction apparatus and the HPLC system used are shown in Fig. 3a. The plasma sample spiked with α-TN and γ-TN was injected into the extraction column. Elution was carried out stepwise with *n*-hexane and methanol-saturated *n*-hexane. Solvents were equilibrated in situ with aqueous plasma. A fraction containing α-TN and γ-TN was monitored with the UV detector and collected, and the solvent was evaporated to dryness. The residue was dissolved in the mobile phase, which was *n*-hexane–ethanol adjusted to give a medium capacity ratio ($k'=5$).

Using a 0.5% ethanol eluent, the α-TN and γ-TN homologues were separated at a resolution of ca. 1.1. No interference was encountered in the determination of α-TN and γ-TN (Fig. 5b). Using relative peak heights with respect to the γ-TN internal standard, the correlation between the peak-height ratio and the amount of sample added to the plasma was found to be linear. The linear regression equation (linear correlation coefficient in parentheses) of the curves was $y=4.66x+0.22$ ($r=0.9997$), where y and x are the peak-height ratio (α-TN/ γ-TN) and the concentration (μg/ml) of plasma, respectively.

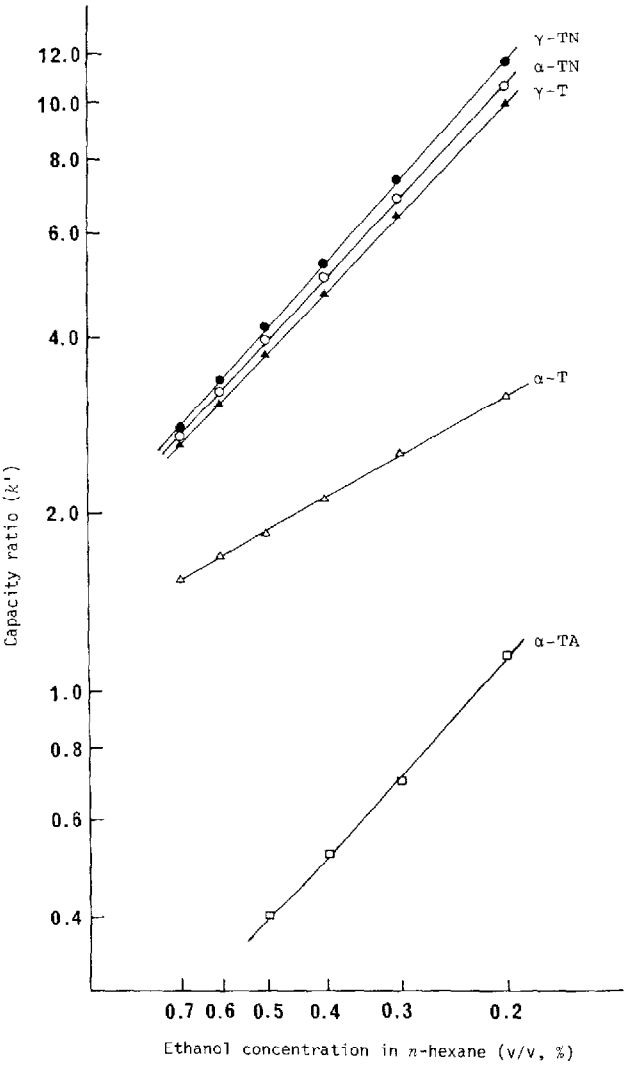

Fig. 4. Solvent composition (%, v/v) versus capacity ratios (k') of tocopherol derivatives. α-TN = α-tocopherol nicotinate; γ-TN = γ-tocopherol nicotinate; α-T = α-tocopherol; γ-T = γ-tocopherol; α-TA = α-tocopherol acetate.

To develop an efficient on-line system, the extraction and separation components were connected with a tube vacuum evaporator [3,4]. The extraction column outlet was connected to the inlet of the evaporator, as shown in Fig. 3a. A portion of the methanol-saturated *n*-hexane was introduced into the evaporator and could thus be evaporated under vacuum. HPLC eluent was added to dissolve the residue in the evaporator. The on-line column-switching procedure was carried out by means of various mechanical valves [3,4,11,12]. This system could be automated by operating these valves electrically.

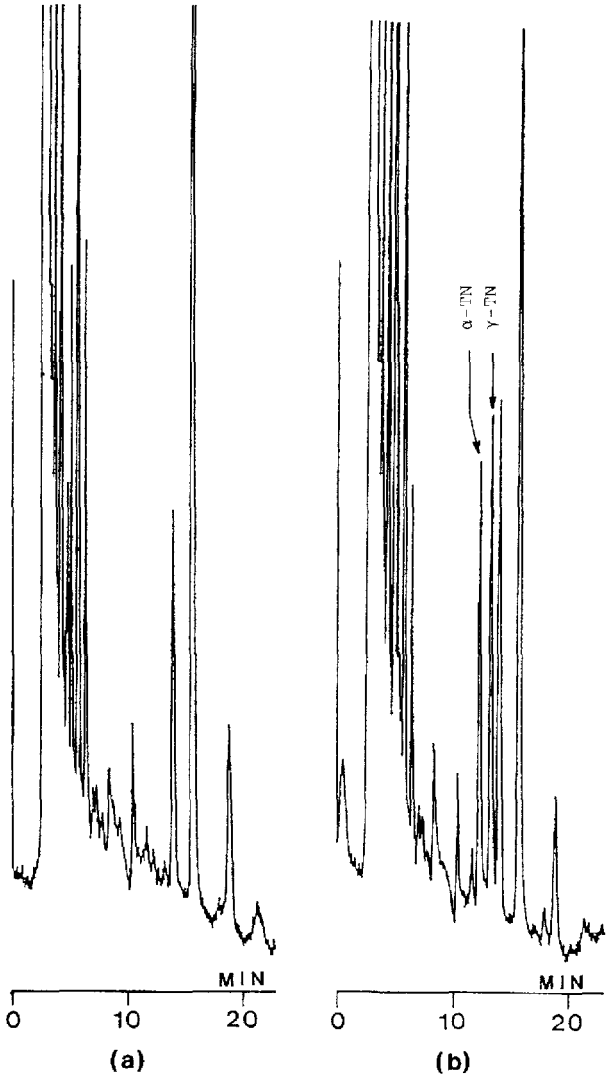

Fig. 5. Chromatograms of a solvent extract from beagle dog plasma (a) without administration, (b) with added I.S. after administration of α-TN. Mobile phase, 0.5% (v/v) ethanol–*n*-hexane; column, LiChrosorb Si 60, 5 μm, 25 cm × 4 mm I.D.; wavelength, 264 nm; sensitivity, 0.001 a.u.f.s.

Direct determination of α-TN in the plasma of beagle dogs and humans

The phase systems and procedures presented in this paper were used to determine α-TN in plasma. Samples were obtained from four beagle dogs and sixteen human subjects every 2 h over a period of 24 h. The phase system developed for the separation of α-TN and γ-TN in the beagle dog plasma was suitable for human plasma. The HPLC pattern is shown in Fig. 5a and b, and the kinetic parameters of α-TN in Table I. The following parameters were obtained: total area under the plasma concentration–time curve (AUC) calculated with a trapezoidal rule, peak plasma levels (C_{max}) and time required for peak development (t_{max}).

TABLE I

KINETIC PARAMETERS FOR PLASMA FROM DOGS AND HUMANS

Values are mean ± S.D.

	AUC (μg/ml h)	C_{max} (μg/ml)	t_{max} (h)
Beagle dog in fasted state*	3.682 ± 2.820	0.188 ± 0.158	4.2 ± 2.6
Beagle dog after food intake**	47.944 ± 18.136	2.931 ± 1.246	3.3 ± 1.6
Human subject after food intake***	1.394 ± 0.796	0.362 ± 0.236	4.9 ± 1.6

*$n = 4$; 100-mg oral dosage of α-TN.
**$n = 4$; 200-mg oral dosage of α-TN.
***$n = 16$; 200-mg oral dosage of α-TN.

Values of AUC and C_{max} reached a maximum 4–5 h after dosing and decreased very slowly thereafter.

Clinical applications of the procedure will be reported elsewhere.

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