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OPTIMIZATION AND VALIDATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF α- AND γ-TOCOPHEROL IN RAT PLASMA AND ERYTHROCYTES

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SUMMARY

An HPLC method for determination of α- and γ-tocopherol in rat blood has been optimized and validated. The optimum means of separation and analysis of tocopherol homologs were selected on the basis of procedures found in the literature; documentary evidence is presented which shows precision and accuracy were satisfactory. Spectrofluorimetric detection was performed after separation on a Separon SGX NH_2 analytical column with *n*-hexane–2-propanol, 97:3, as mobile phase; racemic tocol was used as internal standard. A suspension of erythrocytes was saponified and tocopherols were extracted in the presence of BHT, ascorbic acid, and pyrogallol. Plasma samples were extracted without saponification but with antioxidant protection.

Validation revealed that intra-day and inter-day *RSD* in plasma were 0.78% and 4.15%, respectively, for α-tocopherol and 4.03% and 13.155%, respectively, for γ-tocopherol. Intra-day *RSD* for erythrocytes were 3.75% and 2. 89% for α-tocopherol and γ-tocopherol, respectively. Absolute percentage recovery from plasma and erythrocytes was 99.8 ± 7.5 and 94 ± 9.0, respectively, for α-tocopherol and 100.2 ± 7.5 and 96.9 ± 7.8, respectively, for γ-tocopherol. Limits of detection in plasma and erythrocytes were 0.005 and 0.002 μg mL^{-1}, respectively, for α-tocopherol and 0.002 and 0.0015 μg mL^{-1}, respectively, for γ-tocopherol. Limits of quantification in plasma and erythrocytes were 0.015 and 0.006 μg mL^{-1}, respectively, for α-tocopherol and 0.006 and 0.0045 μg mL^{-1}, respectively, for γ-tocopherol.

INTRODUCTION

One of the most convenient and preferential means of study of changes of antioxidant status in animal tissues caused by chemical and biological factors is determination of vitamin E in plasma and erythrocytes [1–5]. Some authors assumed that erythrocyte vitamin E content would well reflect the antioxidant status of other cells in the body, because erythrocyte membranes are used as a model of the biomembranes of organisms in general [4,5]. The ratio (α-tocopherol in erythrocytes)/(α-tocopherol in plasma) can be regarded as a biomarker of changes in the uptake of vitamin E by most tissues and could better reflect susceptibility to extracellular oxidative stress than the level of α-tocopherol in plasma [5]. γ-Tocopherol has also been recognized as an important antioxidant with unique features that distinguish it from α-tocopherol [6].

A comprehensive review on the HPLC determination of α-tocopherol and related compounds in biological matrices, including plasma and erythrocytes, has recently been published by Ruperez et al. [7]. These authors reported many methodological differences which could effect the effectiveness of analysis. The methodology is usually determined by the form of vitamin E to be analyzed. In general, separation of all tocopherol homologs is easier by normal-phase HPLC system than by the reversed-phase technique [1,7–9]. When only α-tocopherol is to be separated from other tocopherols and quantified reversed-phase HPLC is chosen [7,10,11]. The possibility of determination of the tocopherol content of erythrocytes with or without prior saponification were described by Vattasery et al. [12] and by Gonzalez-Corbella et al. [13], respectively. Vattasery also pointed out that low recovery of tocopherols in some analyses was because of the labile nature of the analyte under oxidizing conditions and that this could be avoided by synergistic use of BHT, ascorbic acid and pyrogallol during the saponification and extraction procedure. Apart from rare reports [14] saponification and antioxidant protection have not been used in plasma analysis.

According to many reports [1,7,8,12] it seems that quantification of the β, γ, and δ homologs of tocopherol and tocopherolquinones could be achieved with satisfactory precision and accuracy only by use of a sensitive detector. For this reason fluorimetric or electrochemical detection are preferred to UV. α-Tocopheryl acetate [8] and tocol [1] are usually used as internal standards, tocol being preferred for normal-phase HPLC. It has been remarked [7] that use of an internal standard has some advantages over use of an external standard, because of better reflection of analyte

loss during sample preparation. This is because of the susceptibility of tocopherols to oxidation.

In our study of the increased susceptibility to oxidative damage of rat erythrocytes in hyperlipidemic subjects in the presence of a variety of environmental pro-oxidants we have sought a simple and convenient method with selectivity sufficient to separate α-tocopherol and γ-tocopherol and with satisfactory recovery especially when determining levels of the analytes in erythrocytes. Among several possibilities we chose normal-phase HPLC with fluorimetric detection. Antioxidant protection and saponification were used for erythrocyte samples and antioxidant protection for plasma samples; that these were the optimum procedures was reflected in good validation data.

EXPERIMENTAL

Chemicals

Tocopherols, butylated hydroxytoluene (BHT), ascorbic acid, and pyrogallol were purchased from Sigma–Aldrich Chemie (Germany) and racemic tocol from Matreya (Pleasant Gap, PA, USA). *n*-Hexane and 2-propanol were of HPLC grade and were purchased from J.T. Baker (Holland). Absolute ethanol was purchased from Aquawit (Poland). Potassium hydroxide and the standard kit for hemoglobin determination were purchased from POCh (Poland).

Preparation of Stock Solutions and Working Standard Solutions

Stock solutions of α-tocopherol and γ-tocopherol were prepared by dissolving each compound in ethanol at concentrations of 10 $\mu g\ mL^{-1}$ and 1 $\mu g\ mL^{-1}$, respectively. Working solutions were prepared by diluting stock solutions in ethanol to furnish concentrations of α-tocopherol and γ-tocopherol in the ranges 0.1 to 8 $\mu g\ mL^{-1}$ and 0.01 to 0.5 $\mu g\ mL^{-1}$, respectively. Racemic tocol (internal standard) solution was prepared to a final concentration of 1 $\mu g\ mL^{-1}$ in ethanol. All standard solutions were prepared no earlier than three days before the experiment and were stored in the dark at 4°C.

Rat Plasma and Erythrocyte Sample Preparation

Fresh blood (approx. 7 mL) was collected from a male Wistar rat, weighing 250 g, into heparinized glass tubes, wrapped with aluminum

foil, and the tubes were centrifuged at 1000 g for 10 min at 4°C. Plasma was then separated and was immediately used for further analysis. The packed erythrocytes were washed with saline (1 M; 3 × 10 mL) then centrifuged at 1000 g for 10 min. Washed red blood cells (RBC) were immediately used for the measurements of tocopherol content and for determination of hemoglobin.

Analytical Procedures

Procedures with antioxidant supplementation and saponification were conducted according to a slight modification of Vattasery's method [13].

Plasma and Erythrocytes Samples – Supplementation with Antioxidants

Fresh plasma (50 μL) or packed erythrocytes (100 μL) were pipetted while vortex mixing into glass tubes, wrapped with aluminum foil, containing BHT in ethanol (0.0025%, *w*/*v*, 2 mL), ascorbic acid (15%, *w*/*v*, 0.2 mL), and pyrogallol (25%, *w*/*v*, 0.2 mL). Plasma and erythrocyte calibration standards for validation were prepared by adding 0.1 mL of each working ethanol solution of *α*-tocopherol and *γ*-tocopherol and 0.1 mL internal standard (racemic tocol) solution in ethanol (1 μg mL^{-1}). Ethanol (0.2 mL) instead of tocopherol standard was added to samples of plasma and erythrocytes analyzed without addition of standard solutions.

Saponification of Erythrocyte Samples

Erythrocyte samples obtained as described above were heated at 60°C for 30 min after addition of potassium hydroxide solution (10%, 1 mL). The tubes were then cooled to room temperature.

Extraction of Tocopherols

Water (1 mL) was added to samples of plasma and erythrocytes, then a solution of BHT in *n*-hexane (0.025%, 2 mL). Tocopherols were extracted into the hexane by vortex mixing for 1 min. The hexane phase was isolated and evaporated under a stream of nitrogen at 40°C by means of a Zymark TurboVap LV evaporator (USA). The residue was re-dissolved in the mobile phase (0.2 mL) and injected on the chromatographic column.

Chromatography

HPLC was performed with a Merck–Hitachi (Germany) L-7100 LaChrom pump, a Shimadzu (Japan) RF-551 spectrofluorimetric detector

equipped with a Merck–Hitachi D-7500 programmable integrator, and a Rheodyne 7725i sample injector fitted with 20-μL sample loop. The chromatographic conditions were as used by Mino et al. [1]. The tocopherols were separated on a 150 mm × 3.3 mm i.d. Separon SGX NH_2 analytical column (Laboratorni Pristroje Praha, Czech. Republic), fitted with a 50 mm × 3.3 mm i.d. precolumn, by isocratic elution with 97:3 (*v*/*v*) *n*-hexane–2-propanol, at a flow rate 0.8 mL min^{-1}. Before use mobile phase was prepared by mixing and degassed by sonication under reduced pressure for 5 min. Fluorimetric detection was performed at the wavelengths λ_{ex} = 298 nm, λ_{em} = 325 nm, with high sensitivity.

Validation

The analytical method for determination of *α*- and *γ*-tocopherol in rat plasma and erythrocytes was validated by determination of selectivity, linearity and range, precision, accuracy, absolute recovery and limits of detection and quantification.

RESULTS AND DISCUSION

Selectivity

The selectivity of the method was determined by comparison of chromatograms of extracts obtained from native plasma and erythrocytes, extracts from samples spiked either with *α*- and *γ*-tocopherol or with a mixture of pure *α*-, *β*-, *γ*-, and *δ*-tocopherols.

As shown in Fig. 1 good separation of tocopherols was achieved by use of the chromatographic conditions described. Retention times were 3.98, 6.75, 7.91, 9.03, and 14.52 min for *α*-, *β*-, *γ*-, and *δ*-tocopherols and tocol, respectively, and changed less than 3% in both intra-day and inter-day analyses. These results are in good agreement with data obtained by Mino et al. [1], taking into consideration the differences in flow rate of mobile phase. Identification was achieved by chromatography of standard mixtures dissolved in mobile phase under the same conditions as biological sample extracts and comparing the retention times of the peaks observed. No significant differences were observed. Increased peak height and area were observed when plasma and erythrocyte samples were spiked with tocopherols (Fig. 1B). Similar chromatograms were obtained from extracts of plasma and erythrocytes.

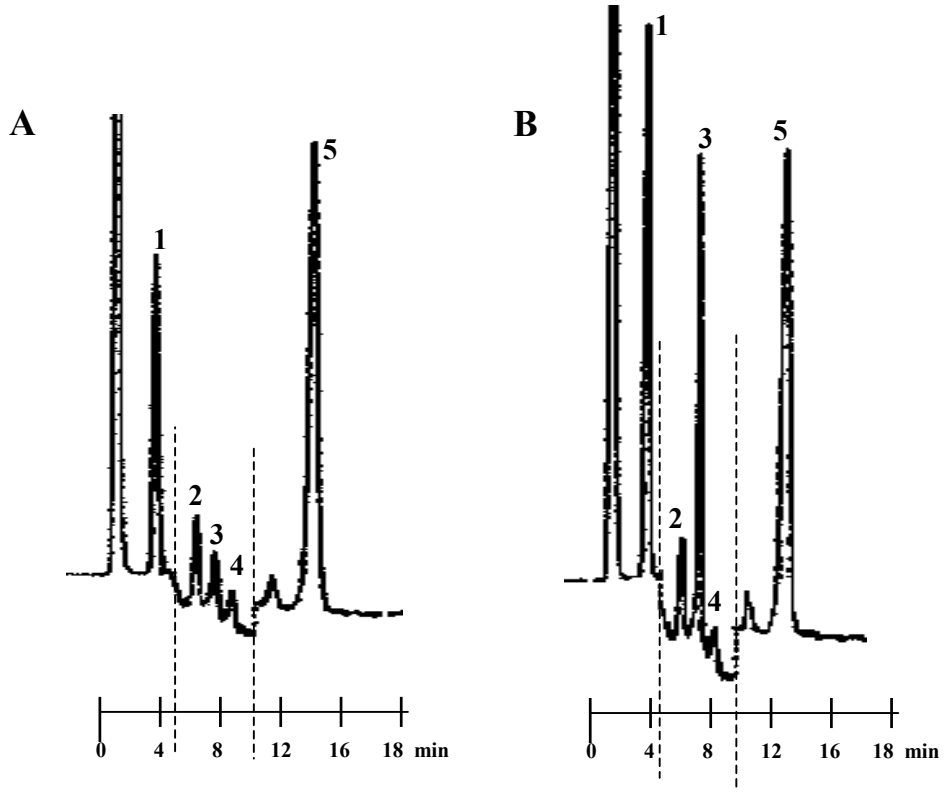

Fig. 1.

Typical chromatograms obtained from tocopherols extracted from 0.1 mL packed erythrocyte sample. A. Native sample extracted after saponification and using antioxidant protection. B. Native sample spiked with 3 μg mL^{-1} α-tocopherol and 0.3 μg mL^{-1} γ-tocopherol and extracted after saponification and using antioxidant protection. Chromatograms were obtained under the same conditions (see Experimental). 1, α-tocopherol; 2, β-tocopherol; 3, γ-tocopherol; 4, δ-tocopherol; 5, tocol (internal standard). Because of the low concentrations of β-, γ-, and δ-tocopherols 32-fold higher attenuation of the plotter was used for these compounds (the parts of the chromatograms between the dashed lines)

Linearity and Range

Standard calibration curves were constructed by spiking plasma and erythrocytes with known amounts of α-tocopherol and γ-tocopherol in the concentration range 0.1 to 8 μg mL^{-1} and 0.01 to 2 μg mL^{-1} respectively. The values obtained for pure samples (without standard added) were subtracted from the values obtained for samples spiked with α-tocopherol and γ-tocopherol standards. In our experiments we prepared calibration curves for each series of assays using pooled fresh blood. The ratios of the

areas of tocopherol peaks to that of the tocol peak were plotted against the concentrations of α-tocopherol and γ-tocopherol. The least square method was used to calculate the regression equations. The terms of calibration curves expressed by the formula $y = ax + b$ for each analytical and quantification procedure are presented in Table I. Because of its very low value, with *RSD* not exceeding 2%, intercept b was omitted from further calculations.

Table I

Calibration curve data and precision of the methods used*

Sample	*n*	α-Tocopherol		γ-Tocopherol	
		a (mean ± *SD*)	*r* (mean ± *SD*)	*a* (mean ± *SD*)	*r* (mean ± *SD*)
Plasma	5	0.3506 ± 0.0040	0.9989 ± 0.007	0.7171 ± 0.0441	0.9991 ± 0.007
Erythrocytes	5	0.8125 ± 0.0388	0.9992 ± 0.0003	1.3879 ± 0.1097	0.9957 ± 0.0054

*Formula of the calibration plot: $y = ax + b$; $b = 0$; peak areas are expressed in integrator units and concentrations are expressed in μg mL^{-1}

Precision and Accuracy

The precision of the assay for tocopherol was evaluated by determining the intra-day and inter-day *RSD* of the measured concentrations of tocopherols in the same samples of plasma and erythrocytes. Plasma and erythrocyte samples were analyzed five times daily (intra-day precision). Plasma samples which were found to be stable after two weeks with no significant change in concentration when stored at –20°C were also analyzed on five consecutive days (inter-day precision). Intra-day assay results only were calculated for erythrocyte samples, because of the significant differences between the concentrations measured even on two consecutive days. Results obtained from these analytical and quantification procedures are presented in Tables II–IV.

The *RSD* values obtained were always lower than 5%, except for inter-day analysis of γ-tocopherol. These data are comparable with data commonly obtained in this method by use of fluorimetric detection [15]. Unexpectedly, *RSD* for γ-tocopherol in plasma was 13.5%. We suppose that this high *RSD* is connected with the very low concentration of γ-tocopherol in rat blood. Even this result fell within limits regarded as acceptable (*RSD* < 15%).

Table II

Precision of the method

Sample	*n*	*α*-Tocopherol				*γ*-Tocopherol			
		Intra-day analysis		Inter-day analysis		Intra-day analysis		Inter-day analysis	
		Concentration (μg mL^{-1}, mean ± *SD*)	*RSD* (%)	Concentration (μg mL^{-1}, mean ± *SD*)	*RSD* (%)	Concentration (μg mL^{-1}, mean ± *SD*)	*RSD* (%)	Concentration (μg mL^{-1}, mean ± *SD*)	*RSD* (%)
Plasma	5	3.379 ± 0.026	0.78	3.497 ± 0.145	4.15	0.024 ± 0.001	4.03	0.023 ± 0.003	13.15
Erythrocytes	5	4.641 ± 0.174	3.75	–*	–*	0.064 ± 0.002	2.89	–*	–*

*Data not obtained because of degradation in erythrocytes during storage (see Experimental)

Table III

Intra-day accuracy of *α*- and *γ*-tocopherol assay in rat plasma and erythrocytes (*n* = 5)

Sample	*α*-Tocopherol			*γ*-Tocopherol		
	Nominal concentration (μg mL^{-1})	Concentration measured (mean ± *SD*, μg mL^{-1})	Recovery (%)	Nominal concentration (μg mL^{-1})	Concentration measured (mean ± *SD*, μg mL^{-1})	Recovery (%)
Plasma	0.2	0.1962 ± 0.010	98	0.02	0.0199 ± 0.006	99
	2	2.0214 ± 0.244	101	0.2	0.1949 ± 0.007	97
	10	10.0644 ± 0.137	100	1	1.0570 ± 0.111	105
Erythrocytes	0.1	0.1096 ± 0.011	109	0.01	0.0115 ± 0.002	114
	1	1.0156 ± 0.121	101	0.1	0.1058 ± 0.031	105
	5	5.1154 ± 0.1645	102	0.5	0.4884 ± 0.013	98

Table IV

Inter-day accuracy of α- and γ-tocopherol assay in rat plasma (n=5)

Sample	*α*-Tocopherol			*γ*-Tocopherol		
	Nominal concentration (μg mL^{-1})	Concentration measured (mean ± *SD*, μg mL^{-1})	Recovery (%)	Nominal concentration (μg mL^{-1})	Concentration measured (mean ± *SD*, μg mL^{-1})	Recovery (%)
Plasma	0.2	0.2213 ± 0.039	110	0.02	0.0230 ± 0.004	115
	2	2.1610 ± 0.404	108	0.2	0.1977 ± 0.017	99
	10	9.9474 ± 0.789	99	1	0.9868 ± 0.0830	99

The intra-day and inter-day accuracy of the assay were calculated by comparing α- and γ-tocopherol concentrations determined in plasma and erythrocyte samples spiked with tocopherols with the corresponding nominal values. The accuracy was expressed as the mean amount (%) of analyte recovered in the assay. Tables III and IV show the intra-day and inter-day accuracy obtained at different concentrations. All the values obtained for accuracy are within limits regarded as acceptable for analysis of biological samples (accuracy 85–115%). The best values were obtained for α-tocopherol in plasma (98–102%), possibly as a result of using anti-oxidant protection in this analysis. In laboratory practice it is extremely difficult to obtain plasma which does not contain traces of hemoglobin, a very potent pro-oxidant which could reduce tocopherol levels during extraction. As is common in such analyses, intra-day results are slightly better than inter-day results and, as might be expected, this is especially so for the α homolog which was present at higher levels.

Absolute Recovery

The mean absolute recovery of the method was calculated in the range of the calibration plots for both compounds. The areas of peaks obtained for native samples were subtracted from values obtained for samples containing tocopherol standards. The absolute recovery was expressed as a percentage of the area of the peak obtained for the pure standard. Values of the mean absolute recovery calculated for each of the procedures used are presented in Table V.

Table V

Mean absolute recovery of α- and γ-tocopherol and tocol (internal standard) in plasma and erythrocytes samples

Sample	Recovery (%, mean ± *SD*)		
	α-Tocopherol	γ-Tocopherol	Tocol
Plasma	99.8 ± 7.5	100.2 ± 7.5	89.9 ± 5.5
Erythrocytes	94.1 ± 9.0	96.9 ± 7.8	84.1 ± 6.0

Limits of Detection and Quantification

Limits of detection for both materials and for two homologs of vitamin E were calculated as the concentration corresponding to a peak three times the noise level on the chromatograms. Quantification limits

were calculated by multiplying the limit of detection by three. Limits of detection were 0.005 and 0.002 μg mL^{-1} for α-tocopherol and 0.002 and 0.0015 μg mL^{-1} for γ-tocopherol in erythrocytes and plasma, respectively. Limits of quantification were 0.015 and 0.006 μg mL^{-1} for α-tocopherol and 0.006 and 0.0045 μg mL^{-1} for γ-tocopherol in erythrocytes and plasma, respectively.

CONCLUSIONS

Validation of the proposed HPLC method for determination of α- and γ-tocopherol in rat plasma and erythrocytes confirmed the behavior of the procedures used. Three aspects of the analysis seem to be critical – choice of a high-resolution analytical column, use of a sensitive detector, especially for assay of γ-tocopherol, and use of antioxidant protection in the extraction stage of the method. Attention to these enables good validation data to be obtained, usually sufficient for animal studies on changes of vitamin E level. Our further experience shows this method could be used directly for determination of tocopherols in human blood and, with slight modification, in other biological fluids and tissues.

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