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DETERMINATION OF THE STEREOISOMERS OF RACEMIC α-TOCOPHEROL IN PHARMACEUTICAL PREPARATIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY

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Abstract: In the present study we used chiral high performance liquid chromatography and gas chromatography to determine the stereoisomers of racemic α-tocopherol. The Chiralcel OD-H and Silar-100 AT columns were used. Statistical evaluation of the results established the precision of these methods.

Keywords: vitamin E, racemic α-tocopherol, HPLC, GC

The term "vitamin E" refers to all the natural and synthetic tocopherols and tocotrienols. They are derivatives of 6-chromanol, which possess a saturated (tocopherols) or triunsaturated (tocotrienols) phytol side chain and differ from one another with respect to the position of the methyl group at the chromanol ring (α-, β-, γ-tocopherols or α-, β-, γ-tocotrienols).

As the molecule, α-tocopherol has 3 chiral centres at 2, 4' and 8' positions and it may be found in the form of 8 optically active isomers (1).

It is often the case that only one of the stereoisomers of an optically active compound shows therapeutic activity, while the others are inactive or even harmful.

In the case of vitamin E, the naturally occurring 2R,4'R,8'R-α-tocopherol (RRR-α-tocopherol) is characterized by the highest biological activity and the greatest practical significance. Natural products, especially food, contain free tocopherols, while commercial ones contain their esters: tocopheryl acetates, succinates and phosphates.

RRR-α-tocopheryl acetate shows the highest biological activity, taken as 100%. The activities of the other isomers range from 21% to 71% (2, 3).

Most pharmaceutical products whose composition includes vitamin E contain racemic all-rac-α-tocopherol or its esters (most commonly acetate). It is, therefore, important to evaluate the stereoisomeric purity of pharmaceutical products containing this compound.

Previous papers described the methods of determining stereoisomers of α-tocopherol in biological material.

The aim of this study was to separate all-rac-α-tocopherol into individual stereoisomers in pharmaceutical products containing (±)-α-tocopheryl acetate using high-performance liquid chromatography (HPLC) and gas chromatography (GC).

Hydrolysis of (±)-α-tocopheryl acetate to α-tocopherol was performed, followed by precolumn derivatization of the standard and the pharmaceutical products with dimethyl sulfate. Derivatization was performed to change the polarity of the analyte by blocking the hydroxyl group to improve the chromatographic properties of the studied compound. Then, the resulting methyl derivatives of tocopherol (α-TMe) were separated by HPLC, yielding 5 peaks corresponding to the 2S (SRS+SSR+SSS+SRR) and RSS, RRS, RRR, RSR stereoisomers. The 2S stereoisomer fraction was collected and the conditions for its separation by GC into 4 stereoisomers: SRS, SSR, SSS, SRR were worked out.

EXPERIMENTAL

Apparatus

Shimadzu liquid chromatograph with the LC-10 AT VP pump, SCL-10 VP system controller, SIL-10 AD VP autosampler, RF-10 AXL fluorescence detector, SPD-10 AV spectrophotometric detector, DGU-14 A degasser and the computer with the CLASS-VP software (version 5.3). Hewlett Packard 5890 gas chromatograph. Gilson FC 203B fraction collector. Büchi R-134 rotavap.

Standards

(±)-α-Tocopheryl acetate (Fluka) 97.3% pure.

Pharmaceutical preparations used in the study

Vitamin E 400 mg capsules (Zentiva), Vitaminum E 400 mg soft capsules (Hasco-Lek).

Reagents

n-Hexane, HPLC-grade and ethyl acetate (Lab-Scan), sulfuric acid min. 95% , anhydrous sodium sulfate and potassium hydroxide, all pure for analysis, (POCH Gliwice, Poland), dimethoxyethane 99.9% (Aldrich), dimethyl sulfate for synthesis (Merck Schuchardt), water, HPLC-grade, ethanol, nitrogen.

Chromatographic conditions

HPLC

Column: CHIRALCEL OD-H 250 × 4.6 mm (Daicel), column temperature: 30°C, injection volume: 200 mL, mobile phase: n-hexane, mobile phase flow rate: 1.5 mL/min, fluorescent detector: λ_{EX} = 295 nm, λ_{EM} = 330 nm.

GC

Capillary column: SILAR – 100 AT 60 m × 0.25 mm, 0.2 mm, column temperature: 165°C, injection port temperature: 260°C, detector temperature: 220°C, injection volume: 1 μL, propellant gas: helium, helium flow rate: 3 mL/min, gas pressure: 60 kPa.

Hydrolysis of α-tocopheryl acetate to α-tocopherol

10 mg of (±)-α-tocopheryl acetate was hydrolyzed with sulfuric acid in ethanol (6 : 44, v/v) and the resulting α-tocopherol was extracted with 50 mL of n-hexane. The n-hexane layer was washed with water and filtered through anhydrous sodium sulfate. The concentration of α-tocopherol in n-hexane was 200 μg/mL.

The same procedure was applied to the pharmaceutical products (the amount of the capsule content corresponding to 10 mg of (±)-α-tocopheryl acetate was weighed).

Derivatization of (±)-α-tocopherol with dimethyl sulfate to α-tocopherol methyl derivatives (α-TMe)

In order to perform derivatization reactions we collected 1 mL of (±)-α-tocopherol standard n-hexane solution obtained from the hydrolysis and samples at the concentration of 200 μg/mL each were prepared. The solutions were evaporated in a nitrogen stream. The dry residues were dissolved in 500 μL of dimethoxyethane. While stirring, 200 mL of 60% aqueous solution of potassium hydroxide and 300 mL of dimethyl sulfate were added drop by drop. The entire mixture was heated for 30 min at 50°C. Following evaporation of dimethoxyethane with nitrogen, the dry residues were dissolved in 1 mL of water. The solutions were extracted twice with 5 mL of n-hexane each. The hexane layers were washed with water and filtered through anhydrous sodium sulfate. The resulting methyl derivatives of the standard (α-TMe) and of the samples at the concentration of 20 μg/mL were injected onto the chromatographic column CHIRALCEL OD-H using n-hexane as the eluent (HPLC method). Five measurements were performed for each sample. The chromatograms are presented in Figures 1- 3. The percentages of stereoisomers are summarized in Table 1.

We also prepared a blind sample: 1 mL of n-hexane was evaporated, the dry residue was dissolved in 500 μL of dimethoxyethane and the procedure described above was then employed.

Collection of the fraction containing methyl derivatives of 2S stereoisomers (2S-α-TMe)

The fraction of 2S stereoisomers containing a mixture of the SRS, SSR, SSS and SRR stereoisomers was collected for 3 min (from minute 11 to minute 14). For each sample (standard, pharmaceutical products) 15 column applications were performed. When the collection of fractions was completed, each sample (the standard and the pharmaceutical products) contained 30 μg of the stereoisomer mixture in the entire collected volume of n-hexane solution (ca. 68 mL). The resulting solutions were evaporated in the rotavap, and the dry residues, after dissolution in 1 mL of ethyl acetate, were injected onto the column AT Silar–100 (filler: poly-bis-cyanopropyl). The concentration of the solutions (the standard and the analyzed samples) was 30 μg/mL (GC method). Three independent determinations were performed for the standard and for the pharmaceutical products.

The recorded chromatograms (for the standard and the samples) are presented in Figures 4 – 6.

Within the same time frame we also collected the blind sample fraction following derivatization, which was also analyzed by GC (Fig. 7).

Gas chromatography was also performed on the solvent used (ethyl acetate) and (±)-α-tocopherol at the concentration of 30 μg/mL (Fig. 8 and 9).

The percentage contents of the stereoisomers in the standard and the pharmaceutical products are summarized in Table 2.

Table 1. Percentage of peak areas for the stereoisomers obtained from derivatization of a-tocopherol with dimethyl sulfate (HPLC method).

Product name	% peak area for the 2S stereoisomers	2R stereoisomers			
		% peak area for the RSS stereoisomer	% peak area for the RRS stereoisomer	% peak area for the RRR stereoisomer %	% peak area for the RSR stereoisomer
Vitamin E 400 mg	51.30 51.41 51.48 52.00 51.57	12.27 12.09 12.00 12.14 12.18	12.30 12.03 12.21 12.00 12.13	12.05 12.10 12.01 11.99 12.02	12.00 11.95 12.15 12.07 12.10
	$\bar{x}$ = 51.55 ± 0.27 RSD = 0.52%	$\bar{x}$ = 12.14 ± 0.10 RSD = 0.83%	$\bar{x}$ = 12.14 ± 0.13 RSD = 1.04%	$\bar{x}$ = 12.00 ± 0.04 RSD = 0.36%	$\bar{x}$ = 12.04 ± 0.08 RSD = 0.63%
Vitaminum E 400 mg	51.20 51.38 51.27 51.40 51.28	12.21 12.19 12.23 12.14 12.18	12.98 12.74 12.48 12.69 12.73	12.01 12.24 12.10 12.12 12.02	11.83 11.93 12.03 11.83 11.91
	$\bar{x}$ = 51.31 ± 0.09 RSD = 0.16%	$\bar{x}$ = 12.19 ± 0.03 RSD = 0.28%	$\bar{x}$ = 12.65 ± 0.12 RSD = 0.95%	$\bar{x}$ = 12.10 ± 0.09 RSD = 0.77%	$\bar{x}$ = 11.91 ± 0.08 RSD = 0.63%
α-Tocopherol standard	52.00 51.41 51.57 51.48 51.61	11.92 12.07 12.03 12.17 12.17	12.13 12.20 12.17 12.27 12.16	12.02 12.45 12.18 12.33 12.14	11.99 11.98 12.07 12.01 12.23
	$\bar{x}$ = 51.61 ± 0.23 RSD = 0.44%	$\bar{x}$ = 12.07 ± 0.10 RSD = 0.87%	$\bar{x}$ = 12.19 ± 0.05 RSD = 0.44%	$\bar{x}$ = 12.22 ± 0.17 RSD = 1.37%	$\bar{x}$ = 12.06 ± 0.10 RSD = 0.86%

Table 2. Percentage areas of peaks 1, 2, 3 and 4 obtained from separation of the fraction of 2S stereoisomers (GC method).

Product name	2S stereoisomers			
	% peak 1 area	% peak 2 area	% peak 3 area	% peak 4 area
Vitamin E 400 mg	2.88 2.90 2.87	27.98 27.96 27.97	66.22 66.20 66.23	2.92 2.90 2.92
	$\bar{x}$ = 2.89 ± 0.02 RSD = 0.74%	$\bar{x}$ = 27.97 ± 0.01 RSD = 0.04%	$\bar{x}$ = 66.22 ± 0.02 RSD = 0.02%	$\bar{x}$ =2.91 ± 0.01 RSD = 0.40%
Vitaminum E 400 mg	3.01 2.98 2.97	26.58 26.60 26.60	68.02 67.99 68.01	3.00 3.02 2.99
	$\bar{x}$ = 2.99 ± 0.02 RSD = 0.70%	$\bar{x}$ = 26.59 ± 0.01 RSD = 0.04%	$\bar{x}$ = 68.00 ± 0.01 RSD = 0.02%	$\bar{x}$ = 2.98 ± 0.06 RSD = 1.97%
α-Tocopherol standard	3.16 3.10 3.19	28.12 28.21 28.20	65.17 65.27 65.29	3.19 3.21 3.27
	$\bar{x}$ = 3.15 ± 0.05 RSD = 1.45%	$\bar{x}$ = 28.18 ± 0.05 RSD = 0.18%	$\bar{x}$ = 65.24 ± 0.06 RSD = 0.10%	$\bar{x}$ = 3.22 ± 0.04 RSD = 1.29%

RESULTS AND DISCUSSION

Of all the chromatographic methods, chiral high-performance liquid chromatography (HPLC) and capillary gas chromatography (GC) were the most commonly used methods for determining α-tocopherol stereoisomers.

In 1991, Vecchi et al. combined these two techniques and separated all-rac-α-tocopheryl acetate into individual stereoisomers. They used HPLC on the chiral phase [(+)-poly(triphenyl methyl methacrylate) bound to silica gel] with the acetonitrile/water (9:1, v/v) mobile phase and got the separation into 2 peaks corresponding to 2R and 2S

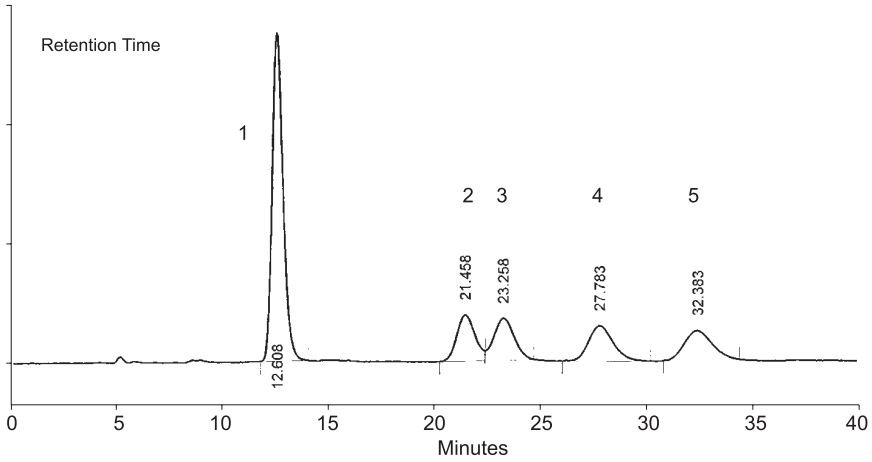

Figure 1. Chromatogram of (±)-α-tocopherol standard after derivatization . Separation by HPLC method into stereoisomers: peak 1 – 2S (SSR+SSS+SRS+SRR) stereoisomers; peak 2 – RSS stereoisomer; peak 3 – RRS stereoisomer; peak 4 – RRR stereoisomer; peak 5 – RSR stereoisomer.

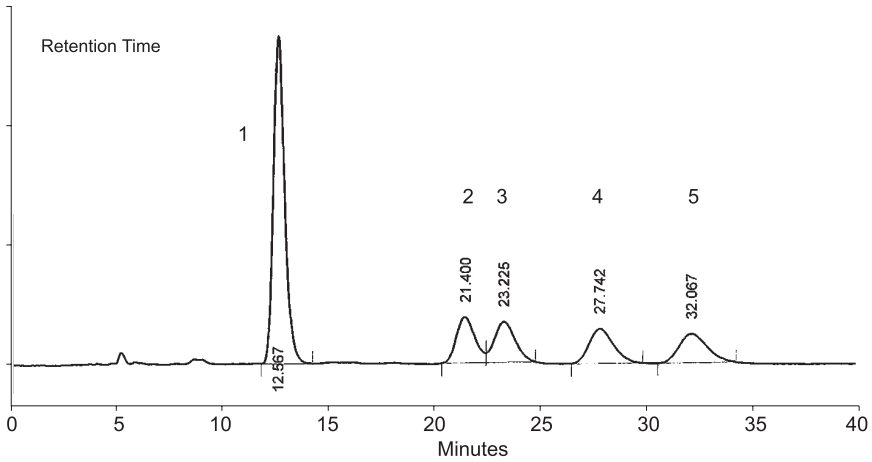

Figure 2. Chromatogram of the pharmaceutical product Vitamin E 400 mg following derivatization. Separation by HPLC method into stereoisomers: peak 1 – 2S (SSR+SSS+SRS+SRR) stereoisomers; peak 2 – RSS stereoisomer; peak 3 – RRS stereoisomer; peak 4 – RRR stereoisomer; peak 5 – RSR stereoisomer.

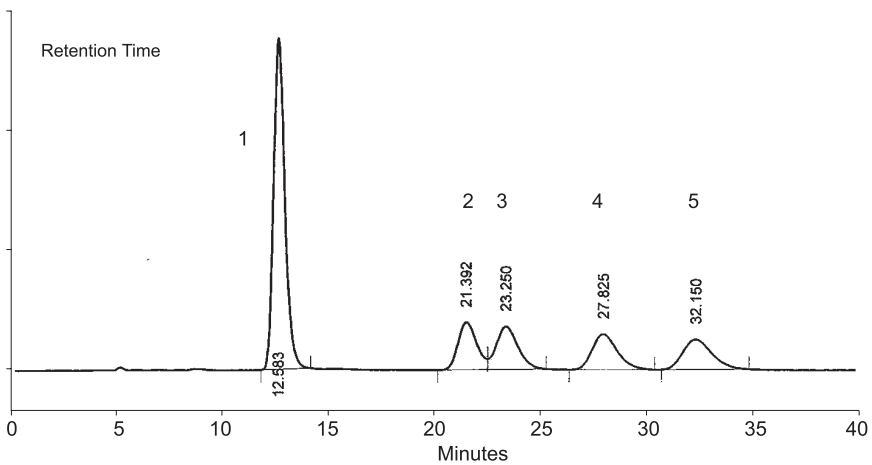

Figure 3. Chromatogram of the pharmaceutical product Vitaminum E 400 mg following derivatization. Separation by HPLC method into stereoisomers: peak 1 – 2S (SSR+SSS+SRS+SRR) stereoisomers; peak 2 – RSS stereoisomer; peak 3 – RRS stereoisomer; peak 4 – RRR stereoisomer; peak 5 – RSR stereoisomer.

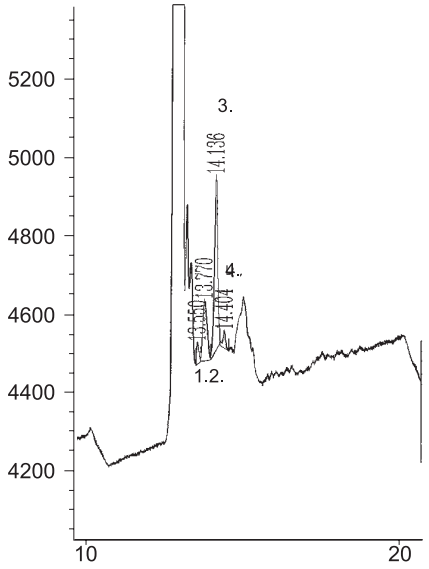

Figure 4. Chromatogram of the 2S fraction of stereoisomers (methyl derivative of the standard). Separation by GC method into 4 peaks: 1, 2, 3 and 4.

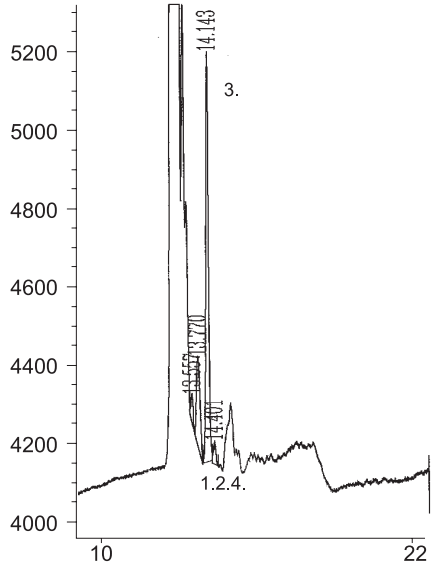

Figure 5. Chromatogram of the 2S fraction of stereoisomers (Vitamin E 400 mg, Zentiva). Separation by GC method into 4 peaks: 1, 2, 3 and 4.

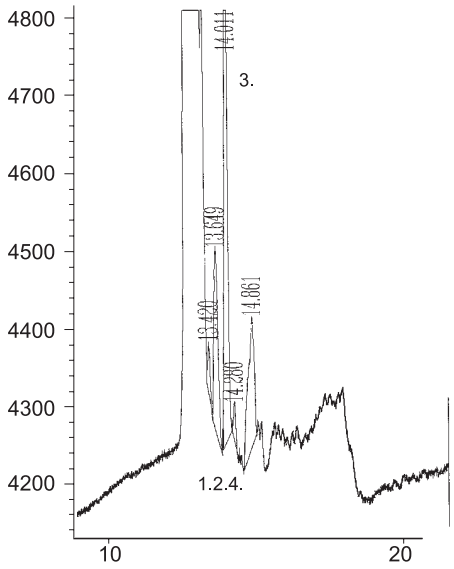

Figure 6. Chromatogram of the 2S fraction of stereoisomers (Vitaminum E 400 mg, Hasco-Lek). Separation by GC method into 4 peaks: 1, 2, 3 and 4.

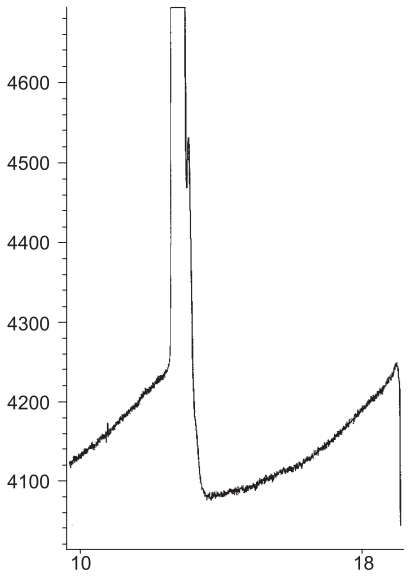

Figure 7. Chromatogram of the blind sample after derivatization.

stereoisomers. They then employed capillary GC to determine 4 stereoisomers in each of the peaks (4).

Good effects of separation of (±)-α-tocopherol stereoisomers on the CHIRALPAK OP(+) and CHIRALCEL OD chiral columns were achieved by other authors, who performed acetylation of the hydroxyl group of α-tocopherol or derivatization with dimethyl sulfate (5-9).

Jensen and Lauridsen also analyzed the stereoisomers of α-tocopherol as methyl ethers by chiral HPLC. Using this method the α-tocopherol stereoisomers were separated into five peaks. The first peak consisted of four 2S stereoisomers, and the

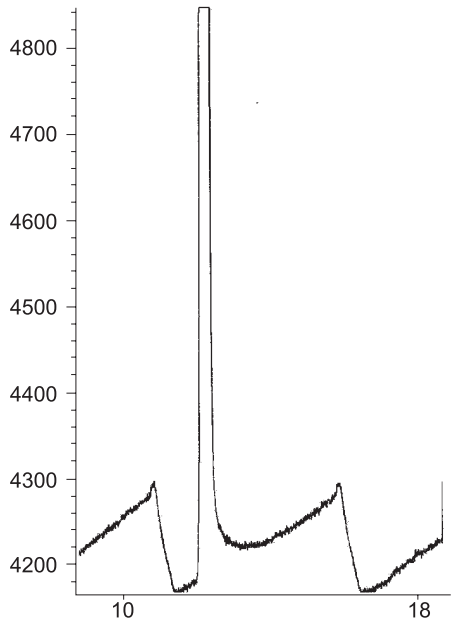

Figure 8. Chromatogram of ethyl acetate.

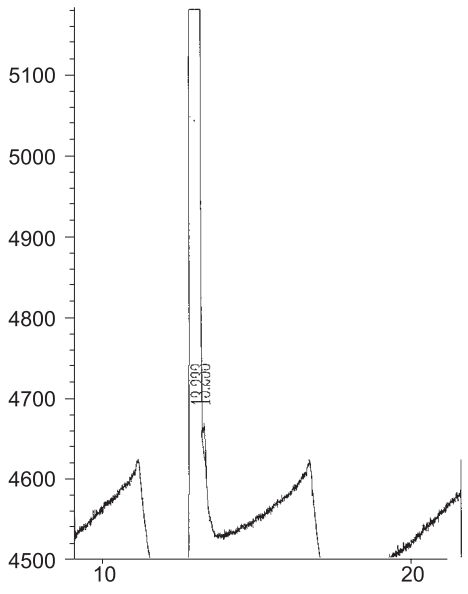

Figure 9. Chromatogram of (±)-α-tocopherol in ethyl acetate 30 mg/mL.

next peaks corresponded to the stereoisomers RSS, RRS, RRR and RSR, respectively (10).

The aim of all the above studies was to determine α-tocopherol stereoisomers in biological material: serum, plasma, rat tissues, using various spectrophotometric, fluorometric and radiometric detectors.

The aim of our study was to develop chromatographic conditions for HPLC and GC, which would allow for separation of the 8 stereoisomers of vitamin E (SRS, SSR, SSS, SRR, RSS, RRS, RRR and RSR).

The first stage involved acidic hydrolysis of (±)-α-tocopheryl acetate to α-tocopherol.

A study on the determination of (+)-α-tocopherol and (-)-α-tocopherol impurities (11) described conditions for the separation achieved after hydrolysis of tocopherol into (+)- and (-)-α-tocopherol on the CHIRALCEL OD-H 250 × 4.6 mm column. The mobile phase consisted in a mixture of n-hexane and isopropanol at the ratio of 99 : 1, v/v (11). Using this phase for the separation of stereoisomers by HPLC on the same column gave poor results. Although the modification of the mobile phase by eliminating isopropanol prolonged the duration of analysis to 40 min, it allowed to achieve a better chromatographic separation of the stereoisomers.

Derivatization of the (±)-α-tocopherol standard and of the samples using dimethyl sulfate was aimed to block the polar hydroxyl group. Introduction of the methyl group into the molecule of α-tocopherol rendered interactions between the analyte and the chiral phase stereoselective and improved resolution of the chromatographic process. The use of fluorescent rather than spectrophotometric detector (λ = 285 nm) increased the sensitivity of the method hundred-fold.

With fluorescent detection, using n-hexane as the mobile phase, 5 peaks were recorded on the chromatograms of the standard and of the samples following derivatization. The individual peaks were identified on the basis of (6), whose authors had at their disposal standards for the individual stereoisomers and employed the same chromatographic conditions. The first of these peaks, with the retention time of 12.6 min, corresponded to the mixture of the following stereoisomers (SRS+SSR+SSS+SRR), and the other four to the stereoisomers RSS, RRS, RRR and RSR with retention times of: 21.5 min, 23.3 min, 27.8 min and 32.4 min, respectively.

A collection (using the Gilson FC 203B fraction collector) of the fraction consisting in the mixture of 2S stereoisomers was time consuming (15 applications for a single analysis of the study sample). Increasing the volume of injection of the studied samples onto the column (over 200 mL) lead to column overload. A better solution would be to use a preparation column with the same filler, which would, however, increase the costs considerably.

Prior to the analysis by GC, the resulting fractions were concentrated by evaporating the solutions in the rotavap. The attempts were to dissolve the dry residues in 1 mL of n-hexane. A better chromatographic image was, however, obtained by dissolving the sample after evaporation in 1 mL of ethyl acetate. The pressure of the propellant gas was set at the optimal level of 60 kPa. The change of this value resulted in a poorer separation of the analyzed peaks. On the chromatograms of the standard and of the samples, 4 peaks with the retention times of 13.6 min, 13,8 min, 14.1 min and 14.4 min were recorded. These peaks had a shorter retention time than the peaks described in (7). This was most probably a result of use of the AT-SILAR-100 column rather than the SILAR 10C column, which is no longer manufactured (Alltech).

In order to establish if any of the peaks did not originate from the solvents, derivatizing solutions or unreacted α-tocopherol, the following were injected onto the capillary column: blind sample following derivatization, ethyl acetate and α-tocopherol 30 μg/mL. In none of the cases peaks with the above retention times were observed. Therefore, the recorded peaks (1, 2, 3 and 4) originate from the following stereoisomers: SSR, SSS, SRS and SRR.

In the study by G. Riss (6), the third separated peak had the greatest area, corresponding to the SRS stereoisomer. In our study, also the area of the third peak was the greatest, which may suggest that the order of the peaks of the separated stereoisomers is also the same (SSR, SSS, SRS, SRR).

The two-stage analysis by HPLC/GC has enabled separation of α-tocopherol derivatives (α-TMe) into 8 stereoisomers. HPLC allowed to separate and identify the following stereoisomers: RSS, RRS, RRR, RSR, while GC enabled separation of only the 2S stereoisomers. However, it was impossible to establish unequivocally which peak originates from which of the stereoisomers: SSR, SSS, SRS or SRR.

Observation of 8 stereoisomers with the same retention times in the pharmaceutical products and in the standard, with their the same percentages, confirm the correct selection of raw materials used for the manufacture of the pharmaceutical products investigated.

The statistical analysis has shown that the precision of both the methods: HPLC and GC is satisfactory. The relative standard deviation was less than 2.0% in both of them.

Hopefully, the progress in the field of new stationary phases will offers an opportunity to separate α-tocopherol into 8 stereoisomers using simpler and more rapid methods, using one chromatographic technique.

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