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HPLC method for simultaneous determination of retinol, α-tocopherol and coenzyme Q_{10} in human plasma

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Abstract

A simple HPLC method with UV detection is proposed for the simultaneous determination of three lipophilic vitamins: all-*trans*-retinol, α-tocopherol and coenzyme Q_{10} (ubiquinone) in human plasma. The following chromatographic conditions were used: RP-18 column, a mobile phase consisted of methanol – *n*-hexane 72:28 (v/v) and UV detector set at 324, 292 and 276 nm for all-*trans*-retinol, α-tocopherol and coenzyme Q_{10}, respectively. The linearity range was 0.35–70 μM for all-*trans*-retinol, 0.23–44 μM for α-tocopherol and 0.12–23 μM for coenzyme Q_{10}. Deproteinised plasma samples were extracted with *n*-hexane prior to the analysis. The within-day and between day reproducibilities were 1.5 and 3.7% for all-*trans*-retinol, 4.0 and 5.8% for α-tocopherol and 2.3 and 3.1% for coenzyme Q_{10}, respectively. Using the proposed method the following recoveries were achieved: 91% for all-*trans*-retinol, 86% for α-tocopherol and 88% for coenzyme Q_{10}. The method was applied to the determination of the levels of retinol, tocopherol and coenzyme Q_{10} in plasma of healthy children and children treated by elimination diet.

Keywords: All-*trans* retinol; α-Tocopherol; Coenzyme Q_{10}; Ubiquinone; Elimination diet; RP-HPLC

1. Introduction

The knowledge about the influence of oxidative stress on human health has increased in the last few decades. Oxidative stress, defined [1] as an imbalance between oxygen free radicals formation and their scavenging by antioxidants, is recognised to play an important contributory role in the pathogenesis of numerous degenerative [1] or chronic diseases such as arteriosclerosis, allergy or cancer [2,3]. Humans along with other aerobic organisms, have evolved a variety of mechanisms to protect themselves from the potentially toxic effects of reactive molecules [1]. The so called antioxidation complex includes enzymes such as catalase and superoxide dismutases, repair enzymes such as DNA glycosylases as well as water and lipid soluble vitamins such as ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), carotenoids, retinol (Vitamin A) and coenzyme Q_{10} [1]. The knowledge of the level of antioxidants in human plasma allows estimating the state of health and can help in treatment of serious diseases. There is a hypothesis that an observed decrease of antioxidant intake in developed countries over the past 30 years and changes in dietary pattern can cause the vulnerability to the oxidative stress [4,5]. Probably, one of the effects connected with the dietary habits is an increase in a number of allergic diseases.

Quantification of lipophilic vitamins in clinical or biological samples is very important from the medical, epidemiological and informational points of view. Simultaneous determination of these compounds in biological samples (plasma, serum, tissues and plant material) appears to be difficult due to their liability to photooxidation, presence of *cis*- and *trans*-isomers and their diverse polarities. Additionally, as they are accompanied by complex matrix, hence their assay requires an intense sample preparation step. For the separation of the fat soluble vitamins various extraction methods were proposed [6].

Several papers were published relating to the determination of lipid soluble antioxidants and vitamins in plasma or tissue samples using various chromatographic techniques. Reversed

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phase HPLC (RP-HPLC) method with UV–vis detection seems to be the most often used technique for the determination of several lipophilic antioxidants such as retinol, γ- and α- tocopherols, lycopene, α- and β-carotenes, Vitamins D_3, K_1, K_2 and others [7–13] in plasma or tissue samples. Recently, RP-HPLC technique with PDA detector was proposed for assay of various carotenoids and tocopherols [14]. Content of retinol acetate, δ-, γ-, α- tocopherols and α- tocopherol acetate in infant formulas was quantified by RP-18 HPLC with UV detection [15]. The same technique was used for assaying Vitamins A and E in milk powder samples after supercritical fluid extraction [16]. The chromatographic behaviour of tocopherols [17] and Vitamin A [18] in micellar medium was the base of their separation and quantification with UV detector in serum [17] and syrup [18] samples. Normal phase liquid chromatography (NP-HPLC) with the use a silica column was applied for determination of carotene, tocopherols and tocotrienols in palm's oil [19]. The native fluorescence of antioxidants was exploited to the determination of tocopherols and tocotrienols in plant extracts [20], chicken meat samples [21] and tissue samples [22] with the use of silica [21] or octadecylsilica [22] columns. The redox properties of lipophilic vitamins were used for their coulometric [23,24] or electrochemical [25] detection after chromatographic separation. Vitamins A and E were determined also by microemulsion electrokinetic chromatography (MEEKC) [26]. Recently, mass spectrometry was used for identification and quantification of α-tocopherol and carotenoids [27], ascorbic acid and carotenoids [28] in botanical samples after separation on RP-18 [27] or silica [28] columns. The intense studies on elaboration of efficient, accurate and precise procedures quantification of lipid soluble vitamins have resulted in a great number of scientific papers which were gathered in comprehensive reviews [29–31]. Among others, only a few chromatographic methods were reported for the simultaneous determination of carotenoids, tocopherols and coenzyme Q_{10}. Hermans et al. [23] have described the method of simultaneous chromatographic determination of these compounds in rat's plasma. Coenzyme Q_{10}, α-tocopherol and cholesterol were separated by coupled LC columns and determined using two detectors: coulometric and UV [32]. Leray et al. [33] have utilised a electrochemical detection for identification and quantification of coenzyme Q_{10}, Vitamin E and products of their oxidation in liver tissue. RP-HPLC was used for the simultaneous determination of tocopherols, ubiquinol and ubiquinone using UV and electrochemical detectors [34]. The electrochemical detection coupled with RP-HPLC system was applied for quantification of ubiquinons, carotenoids and tocopherols in neonatal plasma samples [35]. Greenspan et al. [36] have used cyano-propyl column for separation and quantification some lipid soluble vitamins (e.g. coenzyme Q_{10}, tocopherol and retinol) in dog liver tissues by diode array detector.

As a continuation of our previous work on the level of coenzyme Q_{10} in plasma of patients with inflammatory process [37], we have started an examination of antioxidant's status in plasma of children treated with an elimination diet. There are several papers dealing with the determination of level of various antioxidants in plasma samples of adult patients but only limited information is available about antioxidants level in plasma of children with allergic diseases [38]. As the level of lipid soluble vitamins can be correlated with the status of an antioxidative barrier in human body, α-tocopherol, coenzyme Q_{10} and all-*trans* retinol were selected for our study. All-*trans* retinol was also chosen for investigations as it is one of the additivies (together with above mentioned vitamins) in babies' formulas and supplementary food-stuffs. The study was performed by RP-HPLC technique with UV detection at three wavelengths. The proposed method is discussed from the analytical point of view and applied for determination of the levels of studied antioxidants in plasma of children treated by elimination diet in infancy and early childhood. The dietetic treatment was administered in case of children with diagnosed food allergy or food intolerance.

2. Experimental

2.1. Chemicals and reagents

All reagents used were reagent-grade or better. Methanol and *n*-hexane were HPLC-grade (Baker). Coenzyme Q_{10} (CoQ_{10}) and all-*trans*-retinol (R) standards were purchased from Sigma Chemical Co. (USA). α-tocopherol (α-TP) standard was obtained from Fluka Chemicals Co. (Germany). γ-tocopherol (γ-TP) standard was obtained from Supelco Co. (USA).

Stock solutions of 0.58 mM of CoQ_{10}, 1.20 mM of γ-TP and 1.15 mM of α-TP were prepared by dissolving adequate amounts of reagents in *n*-hexane. Stock solution of R (1.75 mM) was prepared by dissolving an appropriate amount in ethanol. Working solutions of analytes were prepared every day by dissolving appropriate portions of stock solutions in *n*-hexane. All standard solutions were stored at −20 °C.

2.2. Apparatus

Chromatographic system (Thermo Separation) consisted of a 3D detector Spectra System UV 3000, a low-gradient pump P2000, a vacuum membrane degasser SCM Thermo Separation and a Rheodyne loop injector (20 μl). ChromQuest Chromatography Data System software version for Windows NT was used for the acquisition and storage of data. LiChrospher100 RP-18 125 mm × 4 mm (5 μm) column with a guard column 4 mm × 4 mm (5 μm) (Merck, Germany) was used with a mobile phase of methanol – *n*-hexane 72:28 (v/v). The flow rate was 1 ml/min. The wavelengths of the UV detector were 272, 292 and 324 nm.

2.3. Patients

Eighty-eight children treated by diet were selected with randomisation methods. The control group consisted of 56 healthy children on normal diet in infancy and early childhood.

The following criteria were used for the selection of patients into the examined group:

1. The type and length of the elimination diet administered in infancy and early childhood period. The dietetic treatment

Table 1
Characteristics of the tested groups

Group	*n*	Diet followed in infancy and early childhood	Age (in years) mean (S.D.)	BMI mean (S.D.)	The month of life in which the diet started mean (S.D.)	How long the diet was followed (number of months) mean (S.D.)
Studied group	88	Elimination diet	5.24 (1.17)	15.73 (2.02)	3.98 (1.92)	34.58 (16.72)
Control group	56	Normal diet	5.63 (1.13)	16.12 (1.71)	–	–

was prescribed in case of patients with diagnosed allergy or food intolerance. The group of children consisted only of patients with intolerance of cow's milk. Instead of cow's milk they used only one substance, either soya preparations or casein hydrolysates. Their diet started no later than in 6th month of life and was kept at least for 12 months.

2. Age of 2–6 years, because at the age of 2 the lipid metabolism profile becomes stabilised [39].
3. From the studied group were excluded patients who:
   a. suspected of hypertension;
   b. obesity – body weight above 97 percentile;
   c. a history of chronic diseases: glomerulonephritic, diabetes, systemic lupus, chronic diseases of bile ducts and liver with and without cholestasis, infection in the day of acceptance to the study and during the previous 3 weeks, oral administration of glycocorticosteroids.

Table 1 presents the characteristics of the examined children's groups. Permission to carry out the tests was given by the Bio-Ethical Committee at Białystok Medical University. The children's parents signed their agreement, too.

2.4. Sample collection

All blood samples were taken from the elbow flexure, using the minimum stasis. The patients arrived for the tests at least 12 h after their last meal, between 8 and 10 a.m. The blood samples were taken into test tubes with 0.2 ml of 3.8% sodium citrate solution in the proportion of 9:1. Samples of blood were centrifuged at 4 ° C for 30 min. Next the obtained supernatants were frozen at −70 °C and stored for further analysis. Analyzes were made within 1 month after blood collection.

2.5. Sample preparation

The frozen samples were allowed to thaw at room temperature before the analysis. Next, the unfrozen plasma samples were treated using the following procedure: 0.25 ml of plasma was pipetted into eppendorf microcentrifuge tube and deproteinised using 0.5 ml of methanol. After then 0.75 ml of *n*-hexane was added. The mixture was vortexed for 5 min and centrifuged at 5000 rpm for 15 min. Next, the clear hexane layer was transferred to another tube and extraction of plasma vitamins was repeated with new 0.75 ml portion of *n*-hexane. The plasma extracts were combined and evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in 0.25 ml of mobile phase and injected into the HPLC system.

3. Results and discussion

Retinol, α-tocopherol and coenzyme Q_{10} exhibit their characteristic maxima of UV absorption at 324, 292 and 276 nm, respectively. These analytical wavelengths were selected for the HPLC-UV detection to increase the selectivity and sensitivity of analysis. The best separation within a reasonable time as well as good peak shape were achieved with a mobile phase consisting of methanol and *n*-hexane in proportion of 72:28 at a flow rate of 1 ml/min. The retention times were: 2.24 ± 0.01, 2.95 ± 0.03 and 7.47 ± 0.12 min ($n = 10$) for retinol, α-tocopherol and coenzyme Q_{10}, respectively.

To determine the amounts of R, α-TP and CoQ_{10} in biological samples calibration curves were constructed by plotting the peak areas versus the concentration. Linearity was achieved in the concentration range 0.35–70, 0.23–46 and 0.12–23 μM for all-*trans*-retinol, α-tocopherol and CoQ_{10}, respectively. The regression equations were: $y = 7.7 \times 10^{10}x + 10092.1$ ($r = 0.999$) for R, $y = 5.2 \times 10^{9}x + 353.9$ ($r = 0.999$) for α-TP and $y = 1.9 \times 10^{10}x + 12895.3$ for CoQ_{10} ($r = 0.989$), where x – concentration of analyte in mol/l. The detection limits calculated as signal-to-noise ratio equal to 3 were: 1.10 μM for R, 0.78 μM for α-TP, 0.83 μM for CoQ_{10}. The quantification limits were 3.65, 2.75 and 2.98 μM for all-*trans*-retinol, α-tocopherol and CoQ_{10}, respectively. The reproducibility of the presented method was estimated by the analysis of a standard mixture containing 3.50 μM of R, 2.32 μM of α-TP and 1.16 μM of CoQ_{10} 10 times a day for 5 consecutive days. The average coefficients of variation of within-day and between-day assays were respectively 1.5 and 3.7% for R, 4.0 and 5.8% for α-TP and 2.3 and 3.1% for CoQ_{10}. The recovery of studied compounds was checked applying extraction procedure described above using spiked plasma samples. The obtained values were $91\% \pm 4.2$ (mean ± S.D., $n = 9$ for spiked plasma samples) for retinol, $86\% \pm 5.2$ for α-tocopherol and $88\% \pm 3.5$ for coenzyme Q_{10}.

A typical chromatograms of a plasma extract registered at three different wavelengths are shown in Fig. 1a–c.

The application to the real samples demands to check the selectivity of the method. The relatively short retention time of α-TP suggests that the related compound – γ-tocopherol is co-eluted with the main compound. In order to check selectivity, the standard mixture of both tocopherols (23 mM of α-TP and 4.6 mM of γ-TP) was analysed. The performed experiments showed that the difference in retention times of both compounds is high enough (2.83 min for γ-TP and 2.95 min for α-TP) for their efficient separation. Additionally, the natural level of γ-TP is only 20% of α-TP, so the presence of γ-TP does not interfere in determination of Vitamin E in human plasma.

Table 2
Average values of plasma antioxidant vitamins for the tested groups of children

Group	*n*	Retinol [μmol/l] mean (S.D.) [95% CI]	α-tocopherol [μmol/l] mean (S.D.) [95% CI]	Coenzyme Q_{10} [μmol/l] mean (S.D.) [95% CI]
Studied group	88	3.30 (2.06) [2.86–3.74]	29.44 (22.35) [24.65–34.24]	0.39 (0.58) [0.27–0.52]
Control group	56	2.44 (1.68) [1.98–2.89]	21.95 (17.01) [17.35–26.55]	0.48 (0.87) [0.24–0.71]
U Mann–Whitney test *p* (two-tailed)		*p = 0.015*	*p = 0.045*	*p* = 0.492

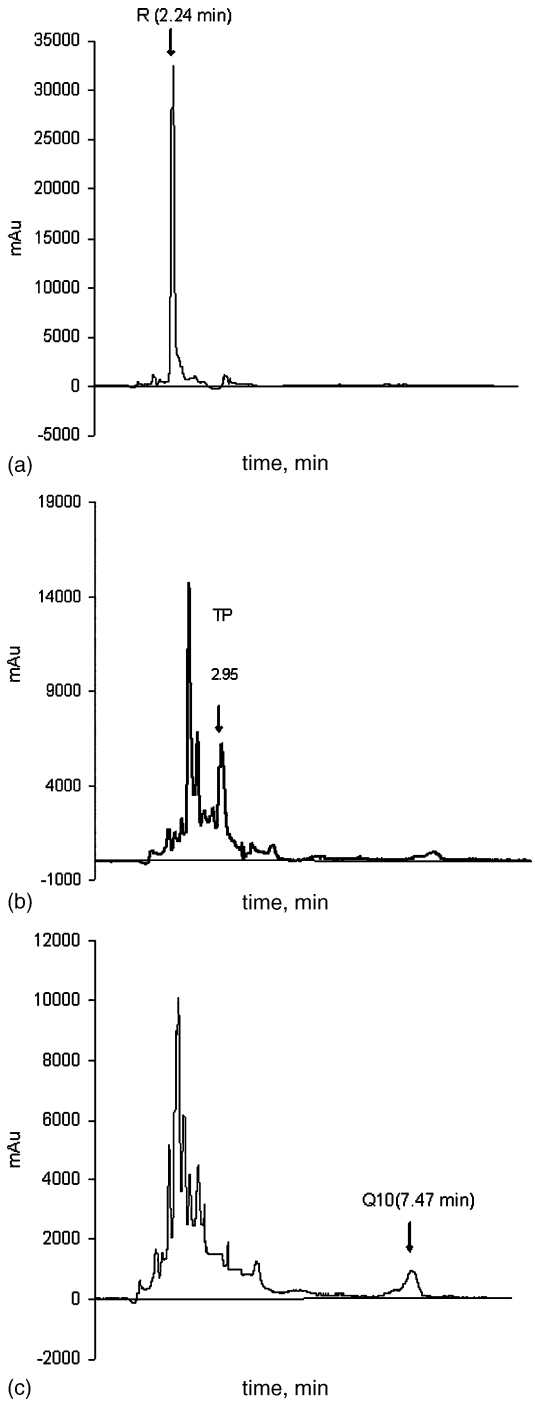

Fig. 1. Chromatograms of plasma extracts recorded: (a) at 324 nm, (b) 292 nm, and (c) 278 nm.

The proposed chromatographic system, in comparison to others similar RP-HPLC systems [33–35] allows the significant reduction of time of single analysis. The fast, simple and reliable procedure minimizes the sample handling and allows the accurate determination of examined compounds.

The described procedure was applied for estimation of level of studied compounds in human plasma. The obtained results are shown in Table 2. Mean serum retinol level of examined group is 3.30 μmol/l (S.D. 2.06) and is higher in statistically important manner than mean retinol level in control group −2.44 μmol/l (S.D. 1.68, at $p = 0.015$). The α-tocopherol level of group under study (29.44 μmol/l (S.D. 22.35)) is also higher than those of control group (21.95 μmol/l (S.D. 17.01)) and the difference is also statistically significant ($p = 0.038$). No difference in serum coenzyme Q_{10} level was observed between examined groups of children. Mean coenzyme Q_{10} concentration is 0.39 μmol/l (S.D. 0.58) for the studied group and 0.48 μmol/l (S.D. 0.87) for the control group ($p = 0.492$).

The observed differences in levels of α-tocopherol as well as all-*trans* retinol can be explained by the influence of administered diet in early period of life due to so-called metabolic imprinting [39]. The examined children were treated by an elimination diet only in period of infancy and early childhood and they are healthy now. The body metabolism adjusted to increased demand of lipid soluble vitamins can remain and last during whole life. Our previous study [37] performed on the group of 43 children (age 1 month–9 years) with acute inflammatory process showed that mean Q_{10} concentration was 0.8 μmol/l (S.D. 0.58). The values obtained in the present study (higher levels of R and α-TP, lower level of Q_{10}), additionally could be caused by good state of health (without any inflammatory process) of examined children. The detailed study on biochemical parameters which have influence on R, α-TP and Q_{10} concentration in children plasma are under preparation and will be published in the future.

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