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3 We propose a simple, reliable and highly sensitive HPLC-micromethod suitable for routine
4 analysis of retinol and α-tocopherol in biological samples.
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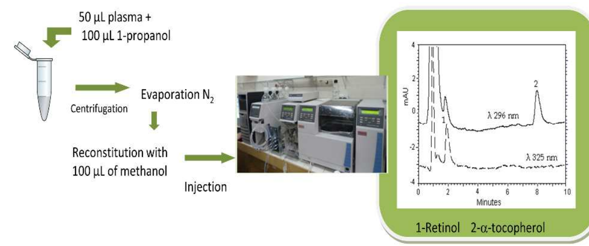

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ARTICLE

Simple microHPLC-UV method for the simultaneous determination of retinol and α-tocopherol in human plasma. Application to intrahepatic cholestasis of pregnancy.

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Manuela. R Martinefski[a], Silvia Scioscia[b], Mario D. Contin[a], Paula Samassa[a], Silvia E. Lucangioli [b,c], Valeria P. Tripodi[b,c†].

Abstract: a) Objectives: The development of a simple, rapid, sensitive, and accurate HPLC-UV method for the simultaneous determination of retinol and α-tocopherol in human plasma, suitable for the evaluation of these vitamins in pregnant women suffering intrahepatic cholestasis of pregnancy (ICP). b) Design and Methods: Chromatographic separation of vitamins was achieved with a microcolumn BDS HYPERSIL C18 (100 mm x 2.1mm, 2.4 µm) using a C18 guard column. The developed and validated HPLC method proposed in this study was applied in the assessment of plasmatic concentrations of retinol and α-tocopherol in normal pregnancy (n=37) and in pregnancy complicated with ICP (n=19). c) Results: The validation of the method showed an excellent linearity for retinol and α-tocopherol (r^2: 0.998 and 0.997), high sensitivity (LOD: 0.02 and 0.11µM, LOQ: 0.07 and 0.36 µM), high recovery (96.0%-104.0% and 99.3%-102.0%), respectively. The method requires low sample volume and all procedure of the sample preparation and analytical determination take less than 20 min to be completed. The evaluation of the results in ICP patients showed that α-tocopherol is significantly diminished compared to normal pregnant women (13.1±2.9 vs 22.4±2.2 µM, $p<0.05$) whereas retinol remains unchanged (1.27±0.21 vs 1.25±0.10 µM). d) Conclusions: A simple, accurate, reliable, selective, highly sensitive and cost effective method suitable for routine determinations of retinol and α-tocopherol in biological samples is proposed. It was possible to determine the decrease of α-tocopherol in ICP patients by this method.

Introduction

Over the past few years, there has been a surge in interest in fat-soluble compounds due to their beneficial effects on human health. Among the fat-soluble group of vitamins, retinol and α-tocopherol (Figure 1) are the major components of the antioxidant system in humans, protecting cell membranes against peroxidation [1-5].

Figure 1

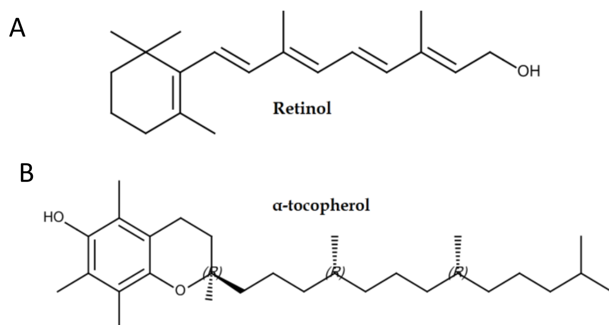

Figure 1: Chemical structures: (A) retinol and (B) α-tocopherol.

Simultaneous determination of these two vitamins in biological matrices appears to be a challenge because of their lability, diverse polarities and the need of sample preparation techniques with multiple steps [6].

Classical sample preparation procedures use several extraction steps with different organic solvents and additives to avoid deterioration of vitamins. As these procedures can be complex, time consuming and tedious, many reports in analytical chemistry use an internal standard method for quantification due to losses of analytes in the extraction step [7-12].

Several chromatographic techniques related to the determination of lipophilic vitamins have been developed and reverse phase HPLC with UV detection has been the most common technique used in both plasma and tissue samples. It is a routine practical instrumentation commonly employed in laboratory because it usually does not require to couple sophisticated equipments for assembling the detection system [11].

Recently, the need for speed, high resolution and sensitivity in bioanalysis has prompted extensive research in the miniaturization of HPLC systems using columns with an internal diameter of 1 or 2 mm instead of 4.6 mm, commonly used.

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In previous works, it was demonstrated that as a result of employing microHPLC systems, analysis time was reduced together with solvent and mobile phase consumptions, less sample requirements were needed, and also sensitivity was increased [14, 15].

As the decrease in lipophilic antioxidants are known to have a contributory role in the pathogenesis of numerous diseases, the precise quantification of vitamins at low levels in biological samples is very important from the medical, epidemiological and informational points of view because not only it allows to estimate the state of health but also it can help in the treatment of several diseases [6].

Intrahepatic cholestasis of pregnancy (ICP) is a specific liver disorder of pregnancy usually benign to the mother though it may have serious consequences for the fetus, such as premature deliveries, fetal distress and perinatal mortality [16, 17]. ICP is characterized by an increment of hydrophobic bile acids in plasma [18] which are known to promote the generation of reactive oxygen species inducing oxidative stress [19-21] and impairing the health of the fetus [22]. Hence, the knowledge of antioxidant vitamins status could contribute in the success of the outcome in this type of patients and to improve the state of health of newborns.

The purpose of the present study is to develop and validate a new simple, accurate, selective, reliable, highly sensitive and cost effective microHPLC-UV method for the simultaneous determination of retinol and α-tocopherol in human plasma. The present method is proposed for the evaluation of plasmatic retinol and α-tocopherol levels in ICP.

Results and discussion

The clinical routine practice need rapid analysis with high sensitivity in a complex matrix, therefore, miniaturization of the column is a good alternative. Reduced diameters of column allow the quantification of vitamins in a shorter time with less consumptions of solvent and sample. In addition, the UV-dual detection makes the analysis of low cost and simple.

Sample preparation and optimization of the chromatographic method

The sample preparation was optimized by evaluating various organic solvents for protein precipitation and extraction of plasmatic vitamins (methanol, acetonitrile, 1-propanol). The advantage of reducing sample volumes respect to conventional procedures was also evaluated.

Determinations of vitamins in plasma are generally based on protein precipitation before liquid–liquid extraction [6-12]. However, protein precipitation with cold 1-propanol followed by evaporation to dryness under a stream of nitrogen resulted to be the best method for sample cleaning. This procedure provides several advantages: high recovery and selectivity and faster separation in a single step compared to other time consuming extraction procedures like two-step liquid-liquid extraction [6-12]. Likewise, it was unnecessary the addition of an internal standard because high recovery is achieved in a single step extraction. Finally, only 50 μL of plasma instead of 200-250 μL of sample volumes usually required for this type of analysis were employed.

Therefore, the proposed method is amenable to be applied in pediatric samples. Currently two reports cite the employment of microvolumes of plasma in pediatry [7, 8]. However, both reports employed a liquid-phase extraction with the addition of antioxidants to prevent the oxidation of vitamins in procedures with extensive exposure time to degradation. Owing to the speed of the purification procedure, the proposed method does not require the addition of antioxidants to the plasma.

To optimize the chromatographic system, it was necessary to evaluate the mobile phase composition by changing the proportion of solvents and flow rates in order to shorten analysis time together with maintenance of adequate resolution. In other methods reported mobile phase was prepared with solvent mixtures like acetonitrile, methanol and ethyl acetate [7], methanol and n-hexane [6], acetonitrile, tetrahydrofurane, methanol and 1% ammonium acetate [8]. Instead, the proposed method employed a simple mixture of methanol: water (94:6, v/v, respectively) considered less toxic and less expensive. Flow rates ranging from 0.7-2.0 mL/min reported by traditional methods [6-12] were reduced to 0.4 mL/min enabling less solvent consumption.

Validation Procedure

Specificity of the method for retinol and α-tocopherol was verified by a good resolution between both vitamins without any interference of the other tocopherols (Figure 2).

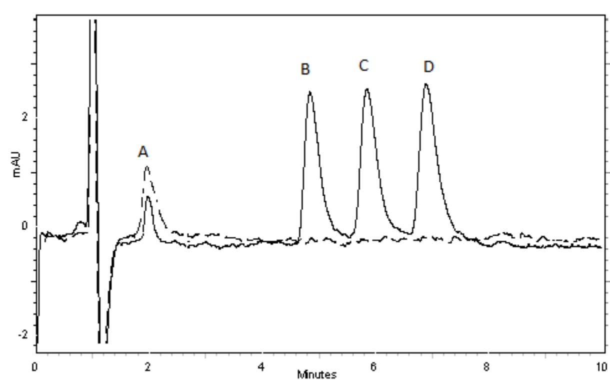

Figure 2: HPLC chromatogram of standard mixture. Retinol and tocopherols were detected at 325 and 296 nm respectively. A: retinol, B:δ-tocopherol, C: γ-tocopherol and D: α-tocopherol. Dash: λ 325 nm and solid line: λ 296 nm.

Linearity was performed at five different concentration levels of each vitamin over the range between 0.1-4.1 μM for retinol and 2.5-74.4 μM for α-tocopherol. Results from statistical analysis showed that the coefficients of determination (r^2) were 0.998 and 0.997 for retinol and α-tocopherol, repectively. The proposed procedure diminished at least 7 times the LOD and LOQ values compared to traditional methods, which LOD values were in the range of 0.3-1.1 μM and 0.8-2.5 μM for retinol and α-tocopherol, respectively (Table 1) [6-10, 12, 13]. Taking into account that the levels of vitamins in human plasma reported for healthy subjects [10, 23-25] are 0.7-3.2 μM for retinol and 7.2-52.0 μM for α-tocopherol, we found that LODs and LOQs values are suitable for their determinations in all types of populations including those with deficiency conditions.

Table 1: Linearity, LOD and LOQ of retinol and α-tocopherol

Analytes	Linear range (μM)	Calibration curve	r^{2a}	LOD[b] (μM)	LOQ[c] (μM)
Retinol	0.1-4.1	y=42178x+246	0.998	0.02	0.07
α-tocopherol	2.5-74.4	y=3707x+35	0.997	0.11	0.36

[a]r^2: coefficient of determination; [b] LOD: Limit of Detection; [c] LOQ: Limit of Quantification

Accuracy was evaluated from recovery studies of spiked plasma samples. Attained recoveries of vitamins in biological samples ranged between 96.2% and 104.0% with acceptable relative

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standard deviation (RSD) values (Table 2). Precision for retinol and α-tocopherol were evaluated for intraday (n=3) and interday (n=9) assays on spiked plasma at upper, middle and lower concentration levels of the calibration curve (Table 2).

Table 2: Precision and accuracy of the method

Vitamin Spiked levels (μM)	*Accuracy* *Recovery %*	*RSD*	*Precision* Intra-day (n=3) RSD	Inter-day (n=9) RSD
Retinol				
0.4	96.2	2.5	1.01	8.14
1.5	100.0	1.0	1.22	2.51
4.1	104.0	1.6	1.43	3.19
α-Tocopherol				
4.9	99.3	3.7	1.09	1.45
15.4	99.5	3.2	1.47	1.62
40.0	102.0	1.3	0.51	3.82

The stability of retinol and α-tocopherol in plasma sample was also evaluated showing no significant loss under the conditions assayed.
The results obtained from the stability study showed that the samples are stable at -20ºC during 1 month and they are apt to be used after being treated by two cycles of freeze-thaw without degradation.

Application

The developed and validated microHPLC method proposed in this study was applied in the assessment of plasmatic concentrations of retinol and α-tocopherol in normal pregnancy and in pregnancy complicated with ICP (table 3 and figure 3). The results of this work show that α-tocopherol is significantly diminished whereas retinol remain unchanged in ICP patients.

Table 3: Comparison of retinol and α-tocopherol in normal pregnant women and ICP patients. Results are expressed as means±SEM. *p<0.05

	Normal pregnant	ICP patients
n	37	19
Retinol (μM)	1.25±0.10	1.27±0.21
α-tocopherol(μM)	22.4±2.2	13.1±2.9*

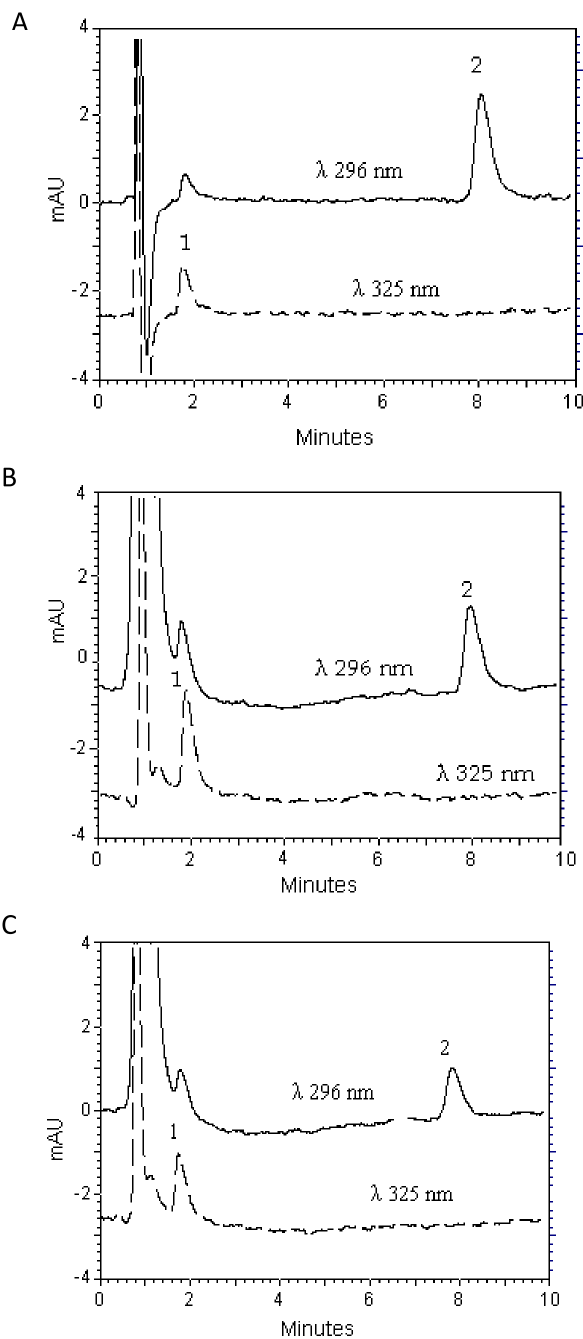

Figure 3: HPLC chromatogram of: A-Standard mixture (retinol 0.39 μM and α-tocopherol 14.9 μM). B- Healthy pregnant woman (retinol 1.82 μM and α-tocopherol 29.2 μM). C- ICP patient (retinol 1.13 μM and α-tocopherol 16.4 μM). 1-retinol and 2- α-tocopherol. Dash: λ 325 nm and solid line: λ 296 nm.

Although in this work, levels of retinol and α-tocopherol were reported in ICP, this method could also be applied for the investigation of retinol and α-tocopherol in the study of pathologies related to vitamins deficiency. In this sense, complex matrices other than plasma can also be evaluated if modifications of the proposed sample pretreatment are carried out.

Experimental

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Materials and methods

Reagents: Retinol used as standard was retinyl acetate and it was provided by Sigma Aldrich (549096 IU/g). α-tocopherol (99.9%), δ-tocopherol (95.5%) and γ-tocopherol (99.1%) were provided by Supelco (Bellefonte, USA). Methanol , n-hexane and ethanol were supplied by Sintorgan (Buenos Aires, Argentina), 1-propanol was purchased from J.T. Baker (Xalostoc, Mexico) and pyrogallic acid and potassium hydroxide from Anedra (Buenos Aires, Argentina). All chemicals employed were HPLC grade. Ultrapure water was obtained by an EASY pure RF equipment (Barnstead, Dudubuque, IA, USA). All solutions were filtered through a 0.45μm nylon membrane (Micron Separations Inc., Westboro, Ma, USA) and degassed before use.

Instrumentation and chromatographic conditions: The Spectra System HPLC assembled equipment was a Thermo Scientific SCM1000 Quaternary pump, P4000 degasser, AS3000 autosampler and UV2000 Dual λ Absorbance detector. Analysis of chromatograms were carried out using a ChromQuest Chromatography Data System software. Separation of vitamins was achieved with a microcolumn BDS HYPERSIL C18 (Thermo Scientific) (100 mm x 2.1mm, 2.4 μm) with a C18 guard column.
Chromatographic conditions were as it follows: an injection volume of 10 μL, a column temperature of 25ºC with an isocratic mobile phase prepared with a mixture of methanol: water (94:6, v/v) and a flow rate of 0.4 mL/min. UV-detection was performed at two wavelengths (λ): 325 nm and 296 nm for retinol and α-tocopherol, respectively. Chromatographic separation of retinol and α-tocopherol in plasma samples was accomplished in less than 10 min.

Preparation of Standard Solutions 25.0 mg of retinol acetate was weighed and added to an alcoholic solution of potassium hydroxide followed by pyrogallic acid. Saponifycation was carried out at 80 °C for 30 minutes; the residue was extracted with n-hexane and conveniently diluted to 4.0 μM retinol in methanol. The accurate concentration of retinol in the stock standard solution was obtained by spectrophotometry at 325 nm, $\varepsilon^{1\%,cm-1}$:1835 [26]. A calibration curve at concentration levels of 0.1-4.0 μM of retinol in methanol was prepared from the stock solution.

The accurate concentrations of stock solutions of α, γ and δ-tocopherol (1mg/mL) were spectrophotometrically determined from the coefficients of extinction of the molecules $\varepsilon^{1\%,cm-1}$ 3267, 3792 and 3515, respectively [27]. Stock solutions were conveniently diluted in methanol. An α-tocopherol calibration curve was prepared at concentration levels of 2.5 -74.0 μM from stock solutions. Working solutions of γ (8.2 μM) and δ-tocopherol (11.5 μM) were also prepared for specificity control.
All solutions were stored at -20ºC and protected from light in amber bottle until use.

Patients This study was carried out in normal pregnant women and patients with ICP at the "Hospital Jose de San Martin" from the Government of the City of Buenos Aires, associated with the University of Buenos Aires, Argentina. It was performed according to the principles of the Declaration of Helsinki and it was approved by the Institutional Review Board and the Bioethical Committee of our Institution. Written consent was obtained in every case. Healthy pregnant women (n=37) and ICP patients (n=19), both groups in the third trimester of pregnancy, were studied.
Heparinized blood samples were obtained after a fasting period of 10 hs and aliquots were immediately processed and centrifuged at 2,000 g for 10 min. Plasma was collected, placed in a capped polypropylene tube, protected from light and immediately stored at -80ºC until analysis.

Sample preparation Before the analysis, the samples were allowed to thaw at room temperature. Firstly, 50 μL of plasma were mixed with 100 μL of cold 1-propanol, stirred with vortex for 2 min, and centrifuged at 9,000 g during 4 min at 25 ºC to spin down the protein precipitate and finally the organic layer was evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in 100 μL of methanol and injected into the equipment. Sample preparation was completed in 10 min.

Quantification and Validation of the Method The quantification of retinol and α-tocopherol was performed using a five points calibration curve ranging from 0.1- 4.1 μM for retinol and 2.5 -74.4 μM for α-tocopherol. Validation of the developed HPLC method was performed according to international guidelines [28]. The parameters considered were: specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and stability. Specificity of the current method was determined from the separation of both vitamins and evaluation of interferences by spiking plasma samples with solutions of γ-tocopherol and δ-tocopherol. The retention time of retinol and α-tocopherol in a combined standard solution was compared to confirm the presence of retinol and α-tocopherol in real samples. The linearity of the chromatographic method was determined by linear least-square regression analysis (n=9). Values of LOD and LOQ in plasma were calculated at signal-to-noise ratios of 3:1 and 10:1, respectively.
Accuracy was evaluated by means of a recovery assay. The recovery assay was carried out by spiking plasma samples with both vitamins at three different levels by triplicate. The three spiked concentrations used in the assays for precision and accuracy were studied at high (4.1 and 40.0 μM), low (0.4 and 4.9 μM) and the middle point levels (1.5 and 15.4 μM) of retinol and α-tocopherol, respectively.
Precision was evaluated on the basis of intraday (n=3) and interday (n=9) analysis repeatability on spiked plasma samples at three different levels already mentionated. Results were expressed as RSD.
The stability of retinol and α-tocopherol in plasma samples was evaluated in plasma aliquots stored at -20 °C and -80 °C during 7, 15 and 30 days and during two frozen cycles. The % stability was calculated as it follows: % stability= (St/S0)x100, being St, the concentration of the analyte at t time and S0, the concentration at initial time. Mean values of three determination were obtained in each point [11].

Statistical Analysis Results were expressed as mean±SEM. Shapiro-Wilk´s *W* test of normality was performed. Differences between groups were analyzed by Student's *t*-test and levels of significance were established at $p < 0.05$.

Conclusions

A new simple, accurate and highly sensitive method using microHPLC with UV detection has been developed for simultaneous quantification of retinol and α-tocopherol in human plasma. The advantages of the proposed method are shorter analysis time (full process of the sample, including pretreatment, takes less than 20 minutes), unnecessary internal standard quantification, small sample volumes requirement and less organic solvent consumption. In addition lower LOD and LOQ values respect to traditional methods can be achieved. In further studies, the results of this method applied to ICP patients will be statistically evaluated.

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Notes and references

[a]Department of Analytical Chemistry and Physicochemistry, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina.

[b]Department of Pharmaceutical Technology, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

[c]Consejo Nacional de Investigaciones Científicas y Tecnológicas, CONICET, Argentina

†**Corresponding author:** Prof. Dr. Valeria Tripodi, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Junin 956 (1113), 5th floor, Buenos Aires, Argentina**.** Tel/Fax: +54 11 4964 8200 (int. 8267), vtripodi@ffyb.uba.ar

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