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Direct liquid chromatography method for retinol, α- and γ-tocopherols in rat plasma

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Abstract

An HPLC method for Vitamins A and E in rat plasma has been developed. The main goals of the method are the small amount of sample, 50 μl, and the direct extraction of analytes in one step with acetone, which is a solvent compatible with the reverse-phase mobile phases. Recoveries, as compared with classical and more tedious methods, were near 100%. The method employs a Supelco Discovery® C18 column and methanol/water (95:5, v/v) as mobile phase. After being developed, the method was validated following ICH guidelines, with UV, fluorescence and electrochemical detectors. It proved to be selective, lineal, accurate and precise. This method greatly simplifies sample treatment and that is a critical point when working with a large number of samples.

Keywords: Retinol; Tocopherols; Vitamins

1. Introduction

Nowadays, it seems quite superfluous to begin an analytical paper addressed to researchers in the area, by emphasising the properties and interest of Vitamins A and E. There are many compendium articles on the subject [1–6]. The efficacy of Vitamin E, alone or with carotenes, in reducing the risk of heart disease, ageing, cancer, cataracts, and other health-related issues has been investigated primarily through epidemiological studies of large populations [7,8].

Nevertheless, many unanswered questions about metabolism, bioavailability or nutrient intake of these vitamins for optimal health leave a tremendous amount of work to do and an important part of it is developed with rats as experimental animals.

This kind of work involves many samples to be measured in order to obtain reliable conclusions and that is why the development of more rapid and reliable analytical tools is of great interest.

Our work group recently published a review [9] which includes well referenced tables that provide in-depth summaries of methodology for the sample pre-treatment and the chromatographic analysis of α-tocopherol and related compounds in foods, pharmaceuticals, plants, animal tissues and other matrices.

Sample treatment is a critical step in the analytical process. Manipulation is time consuming, expensive and the main source of errors and, therefore, it should be kept at a minimum if possible. Therefore, our research trends have then naturally focused on these critical steps.

Vitamins A and E are not chemically bound to proteins, lipids or carbohydrates [10] and using harsh reagents and conditions to free them up (e.g. strong saponification) does not seem necessary and can destroy the vitamins. However, if adequate conditions are not used to release the vitamin from lipophilic milieu, recoveries will be poor, because Vitamin E could be associated with other matrix components and must be freed up in the sample preparation steps.

α-Tocopherol is the most active and abundant isomer of Vitamin E in human plasma, γ-tocopherol is the second one and many studies are being conducted to elucidate its special functions [11].

The objective of the present work was the simplification of sample treatment for Vitamin A and E measurements in rat plasma by HPLC and validation of the new conditions with the detection systems commonly used for this purpose: UV, fluorescence and electrochemical detectors.

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2. Materials and methods

2.1. Instrumentation

Two HPLC systems from Beckman-Coulter (Fullerton, CA, USA) were used one of them being provided with a 126 pump, an autosampler (502e model), a Gold System data processor, a 168 diode array UV detector and a Waters 474 (Milford, MA, USA) fluorimeter. The other one was provided with a 116 pump, an autosampler 507e, a 166 UV detector with variable wavelength and a BAS L4C (West Lafayette, IN, USA) amperometric detector. The chromatographic analysis was performed on a 5 μm particle Supelco Discovery® C18 (Bellefonte, PA, USA) column (15 cm × 0.46 cm) in a Bio-Rad (Hercules, CA, USA) or Gecko-2000 (Cluzeau Info-Labo, Ste. Foy La Grande, France) column oven at 40 °C. For the reference method, tubes were centrifuged with a Megafuge 1.0 R (Heraeus Instruments, Langenselbold, Germany) and hexane was evaporated under a N_2 stream with a sample evaporator DRI-BLOCK DB.3D from Techne (Duxford, UK). For the proposed method, samples were sonicated with a probe sonicator UP200S (Dr. Hielscher, Teltow, Germany) and tubes were centrifuged in a Mikro 22 R (Hettich, Tuttlingen, Germany).

2.2. Reagents

All solvents were HPLC grade quality purchased from Scharlab (Barcelona, Spain). α-Tocopherol, Vitamin A and Vitamin A acetate were from Fluka (Buchs SG, Switzerland), γ-tocopherol was from Sigma (St. Louis, MO, USA) and tocol was a kind gift from Roche (Basel, Switzerland). Acetic acid (glacial) was from Merck (Darmstadt, Germany), and sodium acetate and lithium perchlorate were from Panreac (Montcada i Reixac, Spain). Ultrapure water was obtained in a Millipore Milli-Q system (Billerica, MA, USA).

2.3. Animals and plasma sampling

Plasma for method development and validation was obtained from rats (Sprague–Dawley) from our animal quarters. Animals were anaesthetised with ketamine/azepromacine and blood was obtained by cardiac puncture in EDTA. Blood was rapidly centrifuged to separate plasma and immediately kept at −20 °C until the day of the assay.

2.4. Chromatographic analysis

The reverse-phase HPLC mobile phases were methanol/water (95:5, v/v) as eluant, at a flow rate of 2 ml/min for fluorescence and UV detection. A Supelco Discovery® C18 column was utilised. When the electrochemical detector was employed, mobile phases had to conduct electricity and so it was methanol/A (95:5, v/v), A being a 50 mM aqueous acetate buffer prepared with equimolar quantities of the acid and the sodium salt and 150 mM $LiClO_4$ added.

Fluorescence was employed with excitation at 295 nm and emission at 350 nm. UV detection was performed at 340 nm for up to 5 min for Vitamin A detection and at 295 nm from 5 min to the end of the run to detect tocol and tocopherols. Electrochemical detection was accomplished on a glassy carbon electrode at 700 mV versus Ag/AgCl electrode.

2.5. Reference procedure [12]

2.5.1. Stock and working standards

Individual stock solutions of commercial vitamins were prepared in ethanol; and consisted in 8.0 mg/ml α-tocopherol and 0.5 mg/ml for Vitamin A external standards and 1.0 mg/ml for Vitamin A acetate as internal standard. These solutions were stored in aluminium foil-covered containers and kept at −20 °C. On the day of the assay, the working standard solutions were prepared as follows: α-tocopherol and Vitamin A stock solutions were mixed 1:1 (v/v) and 0.125 ml of the mixture were diluted to 25 ml with ethanol. Actual concentrations of the vitamins in the working standard were determined spectrophotometrically at 294 nm ($\varepsilon^{1\%}_{1\,cm} = 71$) for α-tocopherol and at 325 nm for Vitamin A ($\varepsilon^{1\%}_{1\,cm} = 1835$) and the value obtained was employed for quantification. Vitamin A acetate stock solution was diluted 0.5–10 ml with ethanol.

2.5.2. Working standards treatment

In 100 mm × 10 mm glass tubes, 50 μl of the working standard of α-tocopherol and Vitamin A, 50 μl of the working standard of Vitamin A acetate, 150 μl of ethanol, 200 μl of methanol and 200 μl of 5 mM phosphate buffer, pH 7.4, were extracted twice by probe sonication with 1 ml of *n*-hexane, followed by centrifugation at 500 × *g* for 5 min at room temperature. The supernatants were pooled together and evaporated to dryness. The residue was redissolved in 200 μl of methanol.

2.5.3. Sample treatment

Fifty microlitres of the Vitamin A acetate working solution, 200 μl ethanol and 200 μl methanol were added to 200 μl of plasma. Vitamins were extracted twice with 1 ml *n*-hexane by probe sonication and centrifuged at 500 × *g* for 5 min at room temperature. Supernatants were pooled together and evaporated to dryness. The residue was redissolved in 200 μl of methanol.

2.6. Method development

For optimising the extraction conditions several solvents were tested: *n*-propanol, dichloromethane/*n*-propanol (85:15, v/v), diethylether, acetone/isopropanol (85:15, v/v), acetone/SDS aqueous solution (0.5 mM) (85:15, v/v),

n-propanol/SDS (85:15, v/v), diethylether/SDS (85:15, v/v), acetone, SDS, acetone/isopropanol (50:50, v/v).

Recoveries were tested by processing in parallel 2 standards and 10 samples treated with the reference method with hexane and 2 standards and 10 samples treated with the tested solvent. The ratio solvent to sample was also optimised in the same way.

2.7. Proposed procedure

2.7.1. Stock and working standards

Individual stock solutions of commercial vitamins were prepared in ethanol; and consisted of 8.0 mg/ml α-tocopherol, 1 mg/ml γ-tocopherol and 0.5 mg/ml for Vitamin A external standards and 2.0 mg/ml for tocol as internal standard. These solutions were stored in aluminium foil-covered containers and kept at −20 °C. On the day of the assay, the working standard solutions were prepared as follows: α-tocopherol, Vitamin A and γ-tocopherol stock solutions were mixed 1:1:0.4 (v/v/v) and 0.125 ml of the mixture were diluted to 25 ml with acetone.

Actual concentrations of the vitamins in the working standard were determined spectrophotometrically in solutions individually prepared with the same dilution in ethanol and the value was employed for quantification. Tocol stock solution was diluted 0.450–25 ml with acetone.

2.7.2. Working standards treatment

In 0.5 ml Eppendorf tubes, 50 μl of the working standard of α- and γ-tocopherols and Vitamin A, 50 μl of the working standard of tocol, 100 μl of acetone, and 50 μl of 5 mM phosphate buffer, pH 7.4, were mixed by probe sonication followed by centrifugation at 11,180 × *g* for 4 min, at room temperature. The liquid was directly transferred to HPLC microvials.

2.7.3. Sample treatment

Fifty microlitres of the tocol working solution and 150 μl acetone were added to 50 μl of plasma. Vitamins were extracted by probe sonication and centrifuged at 11,180 × *g* for 4 min at room temperature. The clear supernatant was directly transferred to HPLC microvials for analysis.

2.8. Validation

Standards linearity was verified in each case by analysis of duplicates containing 12.5, 25, 50, 75 and 100 μl of the vitamins working solution (16.7 μg/ml for α-tocopherol, 1.04 μg/ml for Vitamin A and 2.08 μg/ml for γ-tocopherol), made up to 0.150 ml with acetone, 50 μl of tocol solution (36 μg/ml) and 50 μl of phosphate buffer. Sample linearity was tested in duplicate containing 12.5, 25, 50, 75 and 100 μl of plasma, 50 μl of tocol solution (36 μg/ml) and made up to 0.2 ml with acetone. These standards and samples were processed as explained earlier.

Recovery was calculated through the linearity range by adding to 25 μl of plasma 0, 6, 13, 25, 37 and 50 μl of the vitamins working solution, 50 μl of tocol solution (36 μg/ml) and 25 μl of phosphate buffer and made up to 0.2 ml with acetone. A standard curve was processed in parallel to calculate recoveries.

Intra- and inter-assay precision was determined by processing two six-sample series, of 50 μl of the plasma on different days. Standards for quantification were prepared from 50 μl of the working solution and treated simultaneously.

3. Results and discussion

3.1. Optimisation of the sample treatment

One of the bottleneck points in tocopherols analysis in plasma is the two steps of extraction followed by evaporation and redissolution. It is time consuming, needs personal manipulation and when a large number of samples have to be measured it is a potential source of errors. Even recently published methods termed as rapid [13] only decrease the water amount in the HPLC mobile phase in order to shorten the run time, but without any simplification of the sample pre-treatment. HPLC analysis is completely automated and saving 2 min is not very important whereas in some cases resolution may be compromised by this change. As generally pre-concentration is not needed, we tested the possibility of extracting the vitamins with a solvent compatible with the HPLC mobile phase that permitted the direct injection of the extract and gave the same recovery as the reference method.

As it was known, the disadvantage of the presence of halogenated solvents was the extraction of the lower phase which is always more difficult and as there were better options, they were discarded. In other cases, proteins were not precipitated, or a clear supernatant was not obtained or two phases did not appear or recoveries were lower than with the hexane method.

Finally, acetone was found to fulfil all the requirements. (1) It was compatible with the HPLC mobile phase. (2) It precipitated the plasmatic proteins. (3) It provided a clear and clean organic phase easy to transfer. (4) It gave a 100% recovery in this first approximation.

Only two work groups in literature have employed extraction procedures that permitted direct injection of the deproteinised sample of human plasma. Sarzanini et al. [14] employed *n*-butanol/ethyl acetate/acetonitrile (1:1:1, v/v/v). They worked with 100 μl plasma and obtained yields of around 98% employing HPLC with coulometric detection. Julianto et al. [15] employed acetonitrile/tetrahydrofuran (3:2, v/v) also in 100 μl of human plasma and obtained 93% recovery using HPLC with UV detection. Nevertheless, in both cases recoveries were calculated only from the spiked sample, where Vitamin E is added in solution and, therefore, more easily recovered.

Table 1
Percentage of recovery of α-tocopherol from samples with different proportion of extractant (acetone)

Acetone:plasma proportion (v/v)	Recovery (%)
3:1	101 ± 5
4:1	98 ± 4
5:1	101 ± 4
6:1	90 ± 8
Reference method	100

The ratio solvent/sample can be decisive in an adequate recovery and precision of the method because, as the solvent is miscible with the aqueous phase, the polarity of the final extraction mixture can change. When ratios acetone to plasma 3:1, 4:1, 5:1 and 6:1 (v/v) were compared with the reference method from 3:1 to 5:1 recoveries were statistically not different from 100% (Student's *t*-test, 95% confidence) for α-tocopherol, while for 6:1 ratio samples were too diluted and the signal was near the limit of detection. Numerical results can be found in Table 1. Pre-validation assay was developed with 3:1 ratio to obtain maximum signal, but problems related to recoveries appeared. Finally, the ratio 4:1 provided the best results. Simultaneously, the volume of sample was decreased with relation to the reference method from 200 to 50 μl which is very interesting when working with small amounts of sample where many metabolites have to be measured, such as in the case of some experimental animals or new-borns. Thus, the final method as described in the corresponding paragraph was quite simply to mix 50 μl of plasma, 50 μl of tocol working solution as internal standard and 150 μl of acetone. The mixture was sonicated, centrifuged and the supernatant injected in the HPLC system. Tocol was chosen as internal standard because it provides response with all the detection systems employed.

3.2. HPLC conditions

Chromatographic conditions were the classical conditions for the analysis of Vitamins A and E [9]. The column was a C18 of new generation of silica, giving higher efficiency than older ones. This stationary phase cannot achieve the separation of γ- and β-tocopherols, but β-tocopherol is usually considered to be negligible in animal tissues, because γ- and α-tocopherols are the main form of Vitamin E in diets [16,17]. That is the reason why the peak has been assigned to γ-tocopherol, although in purity it would be the sum of β- and γ-tocopherol content.

Fig. 1 shows the chromatograms obtained with the four detectors. The two lower chromatograms are UV detectors, the first one is diode array and the second one is UV with monochromator. Profiles are very similar with the peaks corresponding to Vitamin A, tocol and α-tocopherol, while γ-tocopherol is not detectable at the levels found in samples. The third line corresponds to an electrochemical (amperometric) detector. The potential applied was previously optimised to give the maximum sensitivity. Vitamin A does not give signal, γ-tocopherol gave a small signal and tocol and α-tocopherol were clearly measured. The fourth line corresponds to the fluorescence detector with a small peak corresponding to Vitamin A, and tocol and both tocopherols clearly detected.

3.3. Validation results

3.3.1. Selectivity

Selectivity was proved with the diode array detector by comparison of the spectra in different points of the peaks with those obtained with pure standards. Moreover, the electrochemical and fluorescence detectors are more selective by their own mechanism of response.

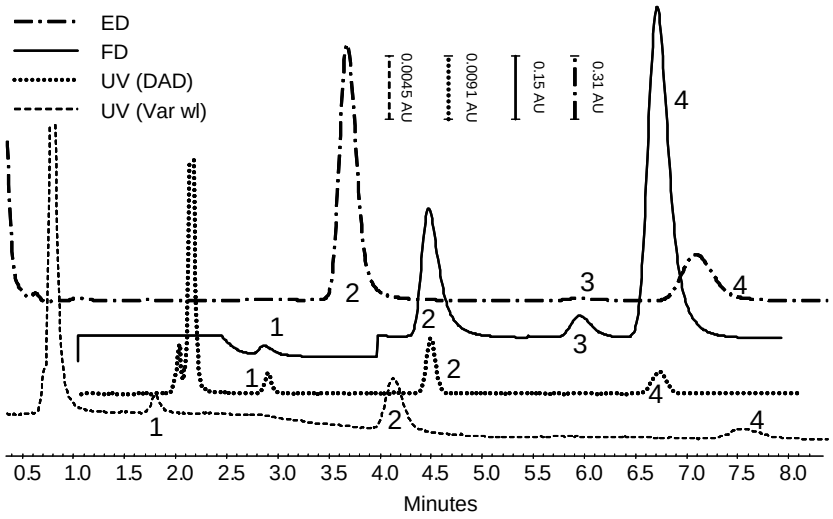

Fig. 1. Representative chromatograms of plasma samples in every detector. ED: electrochemical detector; FD: fluorescence detector; UV (DAD): ultraviolet diode array detection; UV (Var wl): ultraviolet variable wavelength; 1: Vitamin A; 2: tocol; 3: γ-tocopherol; 4: α-tocopherol.

Table 2
Main validation parameters of the method for all the analytes and detectors

	α-Tocopherol				Vitamin A			γ-Tocopherol	
	UV (Var wl)	UV (DAD)	FD	ED	UV (Var wl)	UV (DAD)	FD	FD	ED
Linearity of standards									
Range (μg)	0.189–1.45	0.290–2.22	0.290–2.22	0.189–1.45	0.0065–0.050	0.0051–0.039	0.0051–0.039	0.024–0.19	0.019–0.14
Intercept ± C.I.	−0.02 ± 0.05	−0.01 ± 0.03	−0.1 ± 0.2	−0.03 ± 0.06	−0.01 ± 0.02	−0.010 ± 0.009	−0.002 ± 0.005	−0.04 ± 0.07	−0.001 ± 0.004
Slope ± C.I.	0.80 ± 0.08	0.78 ± 0.04	3.5 ± 0.2	0.8 ± 0.1	11.7 ± 0.5	15.1 ± 0.4	3.1 ± 0.2	10.3 ± 0.9	0.80 ± 0.06
r	0.997	0.9993	0.9990	0.994	0.9990	0.9995	0.998	0.998	0.998
Accuracy of samples									
Recovery (%)	98	95	94	99	98	94	92	102	100
R.S.D. (%)	12	7	6	6	7	5	10	9	5
n	6	6	6	6	6	6	6	6	6
Precision									
Intra-assay									
Mean (μg/dl)	1189	1224	1190	1115	56	34	34	34	60
R.S.D. (%)	3.5	1.8	1.3	2.8	2.0	3.7	2.6	1.8	6.6
n	12	12	12	12	12	12	12	12	12
Intermediate									
Mean (μg/dl)	1252	1174	1144	1204	54	36	36	33	61
R.S.D. (%)	6.7	4.8	4.4	8.6	5.9	7.8	6.5	4.2	6.8

ED: electrochemical detector; FD: fluorescence detector; UV (DAD): ultraviolet diode array detection; UV (Var wl): ultraviolet variable wavelength.

Main validation parameters of the method for Vitamin A, α- and γ-tocopherols, are shown in Table 2. As is well known, Vitamin A gives no response at the electrochemical detector and is poor in fluorescence, while ranges of γ-tocopherol in plasma were too low to be measured in UV.

3.3.2. Linearity

Standards showed a good linearity for all the detectors in the assayed ranges, with correlation coefficients over 0.99 or even 0.999 in some cases, and slopes statistically different from zero. The intercept does include the zero value and, therefore, no bias was found.

3.3.3. Accuracy

Accuracy during method validation was evaluated by the recoveries of the spiked samples and ranged from 92 to 102% with R.S.D. ranging from 5 to 12%. Nevertheless, as previously mentioned, the accuracy was also evaluated during the election of the extraction solvent by comparison of six sample replicates with the classical *n*-hexane extraction method and results were included in Table 1, as mentioned earlier.

3.3.4. Precision

Intra-assay precision showed R.S.D. values ranging from 1.3 to 6.6%. Intermediate precision R.S.D. ranged from 4.2 to 8.6%. The higher variability was due to the electrochemical detector as was expected. In general terms, considering that precision assay includes two different sets of equipment, four different detectors and two different days each, as well as the analysis of labile compounds in a biological sample, precision can be considered very good and this result is mainly due to the simple treatment of samples.

4. Conclusion

Vitamin A and E extraction with acetone in plasma provides quantitative recoveries of these vitamins and it permits the direct injection of the extracts to the HPLC system working in reverse-phase mode. That greatly simplifies sample treatment and, therefore, manipulation and the related problems with the corresponding increase in method precision. Moreover, the method is reliable when applied with UV as well as with fluorescence or electrochemical detectors. Every detector has its own advantages and drawbacks: both UV detectors are easy to use and easily available, moreover, diode array UV detector allows one to obtain the spectra and that is an identification and purity criterion. On the other hand, both detectors suffer from lack of sensitivity and selectivity. Nevertheless, selectivity is usually provided by the chromatographic system and sensitivity is good enough for Vitamin A and α-tocopherol, the most abundant form of Vitamin E in plasma. Fluorescence detector is the best option for tocopherol analysis due to the sensitivity, selectivity and easy handling, nevertheless it is more expensive and the sensitivity for Vitamin A is low. Finally, electrochemical detector is highly sensitive and can be very selective. Its drawbacks are related to the difficulties encountered in daily handling, and Vitamin A gives no response. It would be the option if other related compounds such as α-tocopherol quinone or hydroquinone have to be measured. To sum up, for routine analysis UV detectors are fully satisfactory, and fluorescence and electrochemical detectors provide special properties sometimes necessary for particular samples.

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