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Short communication

Simultaneous determination of vitamins A and E in rat tissues by high-performance liquid chromatography

Coral Barbas*, Mario Castro, Bartolomé Bonet, Marta Viana, Emilio Herrera

Facultad de CC Experimentales y Técnicas, Universidad S. Pablo-CEU, Urbanización Montepríncipe Ctra. Boadilla del Monte, km 5,3, 28668 Madrid, Spain

Abstract

A high-performance liquid chromatographic (HPLC) method to determine vitamins A, Ap and E simultaneously was developed with direct extraction of vitamins from rat tissues with *n*-hexane and probe sonicating. The dry residue was redissolved in chloroform–methanol. Vitamins A and Ap were detected by UV–Vis and vitamin E by fluorescence. Vitamin K, used as internal standard, was detected both by UV–Vis and by fluorescence. Standards and samples were checked for linearity giving correlation coefficients that were higher than 0.99 in the concentration range of 3.1–9.4 for vitamin A, 8.2–24.7 for vitamin Ap and 0.6–1.7 nmol/g in the case of liver extracts and 0.5–3.0 nmol/g in the case of placenta. The intra-assay precision (R.S.D.) varied between 1.48 and 7.25, whereas inter-assay precision was between 4.99 and 7.03. Recoveries ranged between 94±8 and 107±5%. Results from the application of this method to different rat tissues having wide range of vitamin content are presented. © 1997 Elsevier Science B.V.

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1. Introduction

The increasing interest in tissue distribution of certain antioxidant vitamins, including vitamins E and A, arises from their protective role against free radicals. The oxidative stress caused by the net production of these free radicals has been associated to the development of different pathological conditions [1–3]. The beneficial effects of these vitamins in protecting or even preventing those pathological conditions have already been established [4–7]. Determination in small samples, such as foetal tissues of experimental animals and in vitro cell preparations, which are currently being used to study the protection role of these vitamins against oxidative stress [8–12] are within the scope of these studies.

Most of the currently existing methods used to analyze these vitamins include saponification prior to extraction [13–15], a step that we have proved to be unnecessary (unpublished data), since modern analytical methods try to simplify sample pre-treatment steps because they are time consuming and a source of errors [16,17]. To our knowledge, one method has been used to measure retinol and α-tocopherol without saponification but requires enzymatic digestion [18], whereas another one needs clarification of tissue samples with a Sep-Pak C_{18} cartridge to avoid the large solvent peak (acetone) [19]. The methods described measure separately α-tocopherol, retinoids or vitamins A and E, but not vitamin Ap and the only reported method to measure the three of them

*Corresponding author.

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employed two different chromatographic systems [13].

Furthermore, since the amount of vitamins present in different tissues and their responses to UV and fluorescence detection vary, it was considered necessary to include an internal standard in the analytical process. Whereas this standard should have a chromatographic behaviour similar to that of the analytes and should also be capable of being detected and measured both by UV–Vis or by fluorescence detectors, most of the already employed substances (retinyl acetate, α-tocopherol acetate, etc.) lack some of these properties.

2. Experimental

2.1. Instrumentation

A Beckman (Fullerton, CA, USA) HPLC system equipped with a 126 pump, an automatic injector (507e), a 168 diode array detector, a Gold System data processor and a Waters (Milford, MA, USA) 474 fluorimetric detector were used. The chromatographic analysis was performed on a 5 μm particle C_{18} Nucleosil 120 column (15×0.46 cm) kept in a Bio-Rad (Hercules, CA, USA) column oven at 40°C.

2.2. Reagents

All solvents were HPLC grade quality purchased from Scharlau (Barcelona, Spain). Vitamins A, Ap, E and K were from Fluka (Madrid, Spain) and all the other reagents were analytical grade from Merck (Madrid, Spain).

2.3. Animals

Wistar pregnant rats, from our own animal quarter, were decapitated at day 20 of gestation and placenta and fetuses rapidly excised. Then, maternal and fetal liver and fetal brain were immediately dissected and kept at −80°C until processed.

2.4. Methods

2.4.1. Liver

2.4.1.1. Stock and working standards

The following individual stock solutions of commercial vitamins were prepared in ethanol: vitamin A (0.40 mg/ml), vitamin Ap (0.40 mg/ml), vitamin E (8.00 mg/ml) and vitamin K (0.56 mg/ml). These solutions were stored in aluminum foil-covered containers and kept at −20°C. On the day of the assay the concentration of the respective vitamin in the stock solution was determined spectrophotometrically measuring the absorbance of appropriately diluted solutions, actual concentrations were calculated from the wavelengths and molar absorptivities reported previously [20].

Fresh working standards were prepared right before use by pooling and diluting the stock solutions of vitamins A, Ap and E with ethanol, to give final concentrations of 40, 133 and 10.7 μg/ml, respectively.

2.4.1.2. Working standards processing

In 80×12 mm glass tubes, 50 μl of the working standard solution of vitamins A, Ap and E, 50 μl of the vitamin K solution and 2 ml of *n*-hexane were mixed. The mixture was evaporated to dryness in a centrifuge concentrator and the residue redissolved in 200 μl of chloroform–methanol (1:3).

2.4.1.3. Sample processing

In the same type of glass tube described above, 50 mg of tissue were weighed and 50 μl of the vitamin K stock solution were added. Vitamins were extracted twice with 1 ml *n*-hexane by probe sonicator and centrifuged at 2000 *g* for 5 min. Supernatants were pooled together and evaporated to dryness in a centrifuge concentrator. The residue was redissolved in 200 μl of chloroform–methanol (1:3).

2.4.2. Brain and placenta

2.4.2.1. Working standards treatment

50 μl of the vitamin E stock solution (diluted 8 μg/ml with ethanol), 50 μl of vitamin K (the same stock as above) and 200 μl of phosphate buffer (5

m*M*, pH 7.4) were mixed and extracted twice with 1 ml *n*-hexane and treated as described for liver, using chloroform–methanol (1:1) instead of (1:3).

Tissue samples were prepared mixing 50 mg of tissue, 50 μl of vitamin K stock solution and 200 μl phosphate buffer.

2.5. Chromatographic analysis

The reversed-phase HPLC analyses were carried out with methanol–water (96.5:3.5, v/v) as eluent, at a flow-rate of 2 ml/min.

Since the amount of vitamin E present in brain and placenta was very low, detection in the tissue extracts was done by fluorescence. Excitation was set at 295 nm and emission at 350 nm in the first 9 min for vitamin E and at 330 nm and 400 nm until the end of the run (13 min).

Since the amount of vitamins A and Ap in liver was enough for UV, but insufficient for vitamin E, dual detection was carried out. Vitamins A and Ap were measured with UV–Vis at 325 nm, detecting vitamin A in the first 9 min, vitamin K from min 9 to 13 and vitamin Ap from min 13 to 35, while vitamin E was detected with fluorescence as specified above. Analyte concentrations were calculated by the internal standard method.

2.6. Validation

To ensure the homogenicity of samples during the whole test, 0.5 g of brain, 1.0 g of placenta and 2.0 g of liver tissue were separately suspended in phosphate buffer to give a final volume of 4 ml by using a Potter–Elvehjeim-type homogenizer. The use of different amounts of tissue was considered necessary in order to compensate for their respective lipid content, since a high lipid contents difficults both the extraction and the redisolution of the samples. Aliquots were stored at −20°C.

Standard linearity was verified by analysis of two

Table 1
Simultaneous determination of vitamin A and E in rat tissues: validation of the methods

	Liver			Placenta	Brain
	Vit. A	Vit. Ap	Vit. E	Vit. E	
Standard linearity					
Intercept	−0.02±0.02	−0.03±0.04	0.00±0.01	−0.05±0.08	
Slope	0.087±0.003	0.086±0.002	0.24±0.01	1.70±0.05	
r	0.999	0.999	0.999	0.996	
Range (nmol/tube)	3.1–9.4	8.2–24.7	0.60–1.7	0.5–3.0	
Sample linearity					
Intercept	−0.01±0.05	0.02±0.07	0.1±0.2	0.12±0.02	−0.07±0.06
Slope	0.0079±0.0007	0.014±0.001	0.049±0.002	0.0101±0.0001	0.0118±0.0004
r	0.998	0.999	1.000	0.999	0.992
Precision (nmol/g.tissue)					
Intra-assay					
Mean	16.3±0.3	740±30	42±1	27±2	38±2
R.S.D.	1.48	2.97	2.95	7.25	4.52
Inter-assay					
Mean	16.2±0.5	770±30	43±2	27±1	37±2
R.S.D.	4.99	6.60	5.38	5.72	7.03
Accuracy					
% Recovery	94±8	102±4	99±9	101±8	107±5
R.S.D.	5.05	2.38	5.68	9.42	7.11

replicas of 25, 50, 75, 100, 150 and 200 μl samples of the stock solution, prepared as described above and brought to 200 μl with ethanol. Sample linearity was tested in two replicas of 25, 50, 75, 100 and 150 μl of tissue homogenates. All samples were brought to 150 μl with phosphate buffer.

Intra- and inter-assay precision was determined by processing two 6-sample series, of 50 μl of homogenate on different days. Standards were prepared from 50 μl of the stock solution.

Since concentration of vitamins in liver samples was higher, accuracy was evaluated processing two 5-aliquot series of 25 μl of tissue homogenate, to which 0, 12.5, 25.0, 37.5 and 50.0 μl of the standard solution was added. For brain and placenta, 6 aliquots of 50 μl were used and standard additions were of 0, 12.5, 25.0, 37.5, 50.0 and 75.0 μl.

A standard curve processed as described above for linearity was processed simultaneously to sample aliquots with added vitamins to calculate recoveries. Results are expressed as percentage of recovery.

3. Results and discussion

Validation results appear in Table 1 and examples

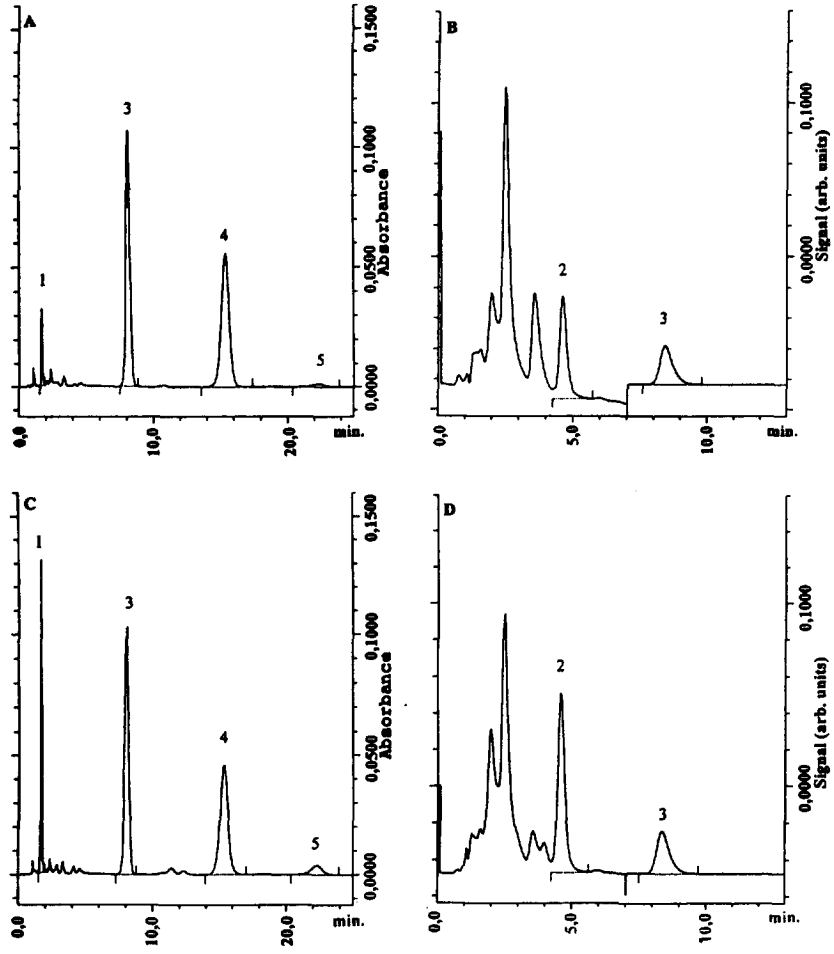

Fig. 1. Chromatograms corresponding to (A) standards with UV detection; (B) standards with fluorescece detection; (C) liver tissue extract (UV); (D) liver tissue extract (fluorescence). Peak identification: 1: vit. A, 2: vit E, 3: vit. K, 4: vit Ap, 5: vit. A est. See conditions in Section 2.4 Section 2.5.

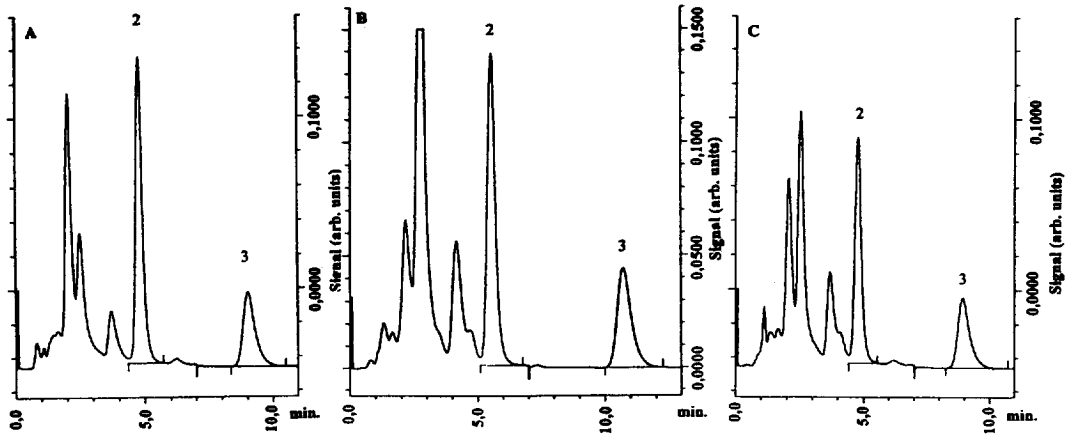

Fig. 2. Chromatograms corresponding to (A) standards; (B) placenta tissue extract; (C) brain tissue extract, all of them with fluorescence detection. Peak identification: 2: vit. E, 3: vit. K.

of chromatograms using standards and actual rat liver, brain and placenta extracts are shown in Figs. 1 and 2. Adequate separation of analyte peaks can be seen. Both standards and samples show a good linearity, with correlation coefficients over 0.99. In both cases, intra- and inter- assay precision and R.S.D. values are low enough for this type of analysis in which the analyte concentrations are below 0.01%. In most cases, R.S.D. values are higher in inter-assay than in intra-assay as could be expected. The recoveries are between 94 and 107%, results that mean good accuracy for the method. The suitability of the internal standard has been probed because none of the analysed tissue samples had an endogenous peak of vitamin K that could interfere with the quantification of this exogenous compound and because vitamin K has both an intermediate chromatographic position and it is easily detectable both in UV and fluorescence.

Among the methods reported for the separation and quantification of these vitamins, methodologic issues (linearity, inter- and intra-assay precision and recoveries) are not always fully evaluated, and sensitivity is normally lower than the one reported here. Our assay was adequate for tissue samples that weighed less than 50 mg including tissues poor in vitamin content as was the case for rat fetuses, while other described methods require samples up to 2 g in mass [21].

Table 2 shows the results obtained with different tissues in the group of rats assayed. They were similar to those already reported by other groups [15,22–27] with the expected variability due to breeds and diets. As can be drawn from these results, the method is capable of measuring high and low vitamin tissue content with a small degree of variation, that could be mainly attributed to animal variability. Concentration of vitamins A, Ap and E always appear lower in fetal than in maternal tissues, which is due to the difficulty in crossing the placental barrier.

The current method of tissue extraction and chromatography is technically straightforward, avoids some of the potential and documented problems using alkaline digestion, and permits the simultaneous measurement of three related vitamins in a variety of tissues. Thanks to its high sensitivity this method may be useful to other groups investigating the metabolism of vitamin E, free vitamin A and stored vitamin Ap in experimental models.

Table 2
Vitamin (Vit.) A, Ap and E content in different rat tissues (nmol of the particular vitamin per g tissue)

Tissue	Vit. A	Vit. Ap	Vit. E
Maternal liver	167.9±10.5	879.6±56.4	58.5±9.6
Placenta	–	–	33.2±4.8
Fetal liver	5.4±0.5	13.1±0.5	5.9±0.5
Fetal brain	–	–	8.8±0.6

Results are means±S.E. for thirteen animals.

References

[1] B. Halliwell, Br. J. Ex. Pathol. 70 (1989) 737.
[2] T.J. Slaga, in Longenecker (Ed.), Nutrition and Biotechnology in Heart Disease and Cancer, Plenum Press, New York, 1995, pp. 167–174.
[3] J.M. Gaziano, J.E. Manson, J.E. Buring, C.H. Hennekens, Ann. NY Acad. Sci. 30 (1992) 249.
[4] M. Viana, E. Herrera, B. Bonet, Diabetologia 39 (1996) 1041.
[5] L. Tesoriere, A. Bongiorno, A.M. Pintaudi, R. D'Anna, D. D'Arpa, M. Livrea, Arch. Biochem. Biophys. 326 (1996) 57.
[6] M.J. Stampfer, E.B. Rimm, Am. J. Clin. Nutr. 62(Suppl.) (1995) 1365S.
[7] H.C.W. Vet, Anticancer Res. 9 (1989) 145.
[8] T. Gaal, M. Mezes, R.C. Noble, J. Dixon, B.K. Speake, Comp. Biochem. Physiol. 112B (1995) 711.
[9] C. Huang, H. Shaw, J. Nutr. 124 (1994) 571.
[10] A. Azzi, D. Boscoboinik, D. Marilley, N.K. Özer, B. Stäuble, A. Tasinato, Am. J. Clin. Nutr. 62(Suppl.) (1995) 1337S.
[11] Y.D. Desai, Methods Enzymol. 105 (1984) 138.
[12] N.R. Badcock, D.A. O'Reilly, J. Chromatogr. 382 (1986) 290.
[13] R. Rettenmaier, W. Schuep, Int. J. Vit. Nutr. Res. 62 (1992) 312.
[14] Q.P. Liu, K.K. Scheller, D.M. Schaefer, J. Anim. Sci. 74 (1996) 2406.
[15] J.-F. Liu, C.-J. Huang, J. Nutr. 125 (1995) 3071.
[16] J.Z. Huo, H.J. Nelis, P. Lavens, P. Sorgeloos, A.P. De Leenheer, Anal. Biochem. 242 (1996) 123.
[17] J.F. Koprivnjak, K.R. Lum, M.M. Sisak, R. Saborowski, Comp. Biochem. Physiol. 113B (1996) 143.
[18] D.W. Nierenberg, S.L. Nann, Am. J. Clin. Nutr. 56 (1992) 417.
[19] H.R. Patel, S.P. Ashmore, L. Barrow, M.S. Tanner, J. Chromatogr. 495 (1989) 269.
[20] The Merck Index, Merck and Co., Whitehouse Station, NJ, 12 ed., 1996.
[21] S. Cadenas, S. Lertsiri, M. Otsuka, G. Barja, T. Miyazawa, Free Rad. Res. 26 (1996) 485.
[22] R.D. Zachman, X. Chen, J. Nutr. 121 (1991) 187.
[23] V.B. Pangala, A. Lacroix, J. Chromatogr. 272 (1983) 269.
[24] T.V. Vliet, F.V. Schaik, J.V. Schoonhoven, J. Schrijver, J. Chromatogr. 553 (1991) 179.
[25] M.K. Konneh, C. Rutherford, E. Anggard, G.A.A. Ferns, Am. J. Clin. Nutr. (Proc. Soc. Exp. Biol. Med.) 210 (1995) 156.
[26] B.J. Burri, R.A. Jacob, Am. J. Clin. Nutr. 47 (1988) 458.
[27] J.K. Lang, K. Gohil, L. Packer, Anal. Biochem. 157 (1986) 106.