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Short communication

Simplified method for vitamin E determination in rat adipose tissue and mammary glands by high-performance liquid chromatography

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Abstract

A method for vitamin E (α-tocopherol) measurement in rat adipose tissue and mammary gland has been developed and validated. Tissues were homogenized in ethanol–water (1:1) and extracted with *n*-hexane. Vitamin K_1 was used as internal standard. Separation was performed by HPLC with methanol–water (96.5:3.5) as eluent in a Nucleosil C_{18} column (15×0.46 cm) at 40°C. Detection was by fluorescence with excitation at 295 nm and emission at 350 nm for vitamin E and at 330 and 440 nm for vitamin K_1. Standards and tissue extracts were checked for linearity giving correlation coefficients over 0.99 in a range of concentrations from 0.56 to 4.51 nmol/g in adipose tissue and from 2.18 to 17.4 nmol/g in mammary gland tissue. Intra-assay precision (R.S.D.) varied between 3 and 4%, whereas inter-assay precision was between 8 and 9%. Recoveries ranged between 95±3% and 98±11% for the two tissues, respectively. Vitamin E was measured in rats that had previously received one oral dose of this vitamin. Whereas vitamin E content in adipose tissue did not differ between late-pregnant and virgin rats, it was significantly higher in mammary gland of pregnant rats, and this difference could be related to the enhanced lipoprotein lipase activity in this group.

Keywords: Vitamins; Tocopherols

1. Introduction

There is continued interest in the role of vitamin E in cellular metabolism, mainly as antioxidant and preventing free radical mediated diseases [1], and a corresponding interest in easy and reliable methods for assessing its concentration in tissues. Epidemiologic studies have shown that α-tocopherol, the most biologically active isomer of vitamin E, levels in blood are very variable, being affected by genetic and lifestyle factors, as well as by other nutrient intakes [2]. Since α-tocopherol is a fat-soluble compound, which is stored in subcutaneous adipose tissue, its concentration in adipose tissue may be a better indicator of its abundance than plasma levels over a relatively long time, because the former is not affected by rapid changes in the level of plasma lipoproteins nor by acute changes in its intake [3].

The mammary gland is an heterogeneous organ with both adipose and connective components that make it difficult to handle for analysis. Nevertheless, the study of its α-tocopherol content is an interesting topic under certain conditions, such as late gestation and lactation, where it becomes the main source of vitamin E for the suckling newborn.

A variety of methods have been described for determination of α-tocopherol isomers. High-performance liquid chromatography (HPLC) separation with fluorescence detection has become the method of choice due to its specificity and sensitivity [4], but since tocopherols are quite sensible to light and air,

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their extraction results in the most critical and time-consuming step in the analytical protocol. Moreover, since tocopherols are fat soluble, their extraction from adipose tissue is difficult. Three different types of extraction methods have been applied: lipid extraction followed by saponification and extraction of the nonsaponifiable lipids [5], direct saponification of the tissue followed by solvent extraction [4,6,7]; or direct α-tocopherol extraction employing sodium dodecyl sulfate (SDS) [8]. It has been shown that saponification causes extensive loss of tocopherols [9] even when working under protective conditions as in the dark and under nitrogen atmosphere, whereas SDS is difficult to clean-up from the chromatographic columns, substantially decreasing their life-span.

A simple method to measure α-tocopherol in heart and liver extracts which avoids saponification and the removal of the coeluting lipids has been described [10], but a normal-phase separation was employed with diol columns prepared in the laboratory. A previously reported method based on the direct extraction in *n*-hexane of α-tocopherol from tissue homogenates by probe sonication, avoiding saponification was applied to liver, brain and placenta [11]. However when applied to adipose tissue or mammary gland, poor recoveries, linearity and reproducibility were obtained due to their high lipidic content. The present study describes a simple way to overcome this problem in rat tissues and consists of carrying out the initial homogenization of the tissues with an ethanol–water mixture. This paper reports the validation of this assay enabling quantification of α-tocopherol in very small samples (50 mg), which could potentially be applied to needle biopsies.

2. Experimental

2.1. Instrumentation

A Beckman HPLC system provided with a 126 pump, an automatic injector (507e model), a Gold System data processor and a Waters 474 fluorimeter was used. Chromatographic analysis was performed on a 5-μm particle C_{18} Nucleosil 120 column (15× 0.46 cm) in a Bio-Rad column oven at 40°C.

2.2. Reagents

All solvents were HPLC grade quality purchased from Scharlau (Barcelona, Spain). Vitamin E and K_1 were from Fluka (Madrid, Spain) and all the other reagents were analytical grade from Merck (Madrid, Spain).

2.3. Animals and tissue sampling

Lumbar adipose tissue and mammary gland samples for method validation were obtained from rats in our animal quarters. Animals were decapitated and tissues rapidly excised and immediately placed in liquid nitrogen and kept at −80°C until the day of the assay. The method was applied to determine α-tocopherol in adipose tissue and mammary gland from virgin and 21-day pregnant Wistar rats. These rats received one oral dose of 10 mg α-tocopherol mixed with 20 mg triolein, 0.5 ml water and 10 mg of Tween 20, 24 h before sacrifice.

2.4. Chromatographic analysis

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

Detection in the tissue extracts was by fluorescence. Excitation was 295 nm and emission, 350 nm in the first 9 min for vitamin E and at 330 nm and 400 nm until the end of the run (min 13) for vitamin K_1.

2.5. Procedure

2.5.1. Stock and working standards

Individual stock solutions of commercial vitamins were prepared in ethanol; and consisted in 8.0 mg/ml vitamin E for external standard and 0.6 mg/ml vitamin K_1 for internal standard. These solutions were stored in aluminum foil-covered containers and kept at −20°C. On the day of the assay working standard solutions were prepared as follows: vitamin E stock solution was diluted 1:250 in ethanol for adipose tissue analysis and 1:100 in ethanol for mammary gland analysis. Actual concentration of the vitamin in the working standard was determined spectrophotometrically at 294 nm and the obtained

value was employed for quantification. Vitamin K_1 stock solution was diluted 1:5 in ethanol for adipose tissue and remained undiluted for mammary gland analysis.

2.5.2. Working standards treatment

In 80×12 mm glass tubes, 50 μl of the working standard of vitamin E, 50 μl of the working standard of vitamin K_1, 50 μl of ethanol and 100 μl of 50 m*M* phosphate buffer, pH 7.4, were extracted twice by probe sonication with 1 ml of *n*-hexane, followed by centrifugation at 2000 *g* for 5 min, at room temperature. The supernatants were pooled together and evaporated to dryness in a centrifuge concentrator (Virtix, USA). The residue was redissolved in 400 μl of chloroform–methanol (1:1, v/v), for adipose tissue, and in 750 μl of chloroform–methanol (1:1, v/v), for mammary gland.

2.5.3. Sample treatment

Approximately 50 mg of either adipose tissue or mechanically grounded powder of mammary gland were exactly weighed and homogenized in a Potter with 10 μl of ethanol–water (1:1) per mg of tissue. A 50-μl volume of the vitamin K_1 working solution was added to 200 μl of tissue homogenate. Vitamins were extracted twice with 1 ml *n*-hexane by probe sonication and centrifuged at 2000 *g* for 5 min, at room temperature. Supernatants were pooled together and evaporated to dryness in a centrifuge concentrator. The residue was redissolved in 400 μl of chloroform–methanol (1:1, v/v), for adipose tissue, and in 750 μl of chloroform–methanol (1:1, v/v), for the mammary gland.

2.6. Validation

Standard linearity was verified in each case by analysis of duplicates of 12.5, 25, 50, 75 and 100 μl samples of the vitamin E working solution (32 μg/ml for adipose tissue and 80 μg/ml for mammary gland), processed as explained above for working standards. Sample linearity was tested in duplicate of 50, 100, 200, 300 and 400 μl of each tissue homogenized, processed as described above for samples.

Intra- and inter-assay precision was determined in both tissues by processing two six-sample series, of 200 μl of the same homogenate on different days. Standards for quantification were prepared from 50 μl of the working solution and treated simultaneously.

Accuracy was evaluated by processing two series of 100 μl of tissue homogenate, to which 12.5, 25, 50, 75 and 100 μl of the standard vitamin E solution were added for adipose tissue and 12.5, 25, 50, 100 and 150 μl for the mammary gland in order to cover the expected range in samples. In all cases volumes are brought up to 400 μl to give a final ethanol–water proportion of 1:1. A standard curve was processed in parallel to calculate recoveries.

3. Results and discussion

Figs. 1–3 show the chromatograms of standards, of adipose tissue and mammary gland extracts, respectively.

Validation results are summarised in Table 1. Both standards and samples show a good linearity, with correlation coefficients over 0.99 in the studied ranges. In adipose tissue a small bias in sample linearity can be observed, and may be explained by the high values of the correlation coefficient ($r>0.999$) that make a very narrow limit of confidence around the intercept.

Intra- and inter-assay precision show acceptable

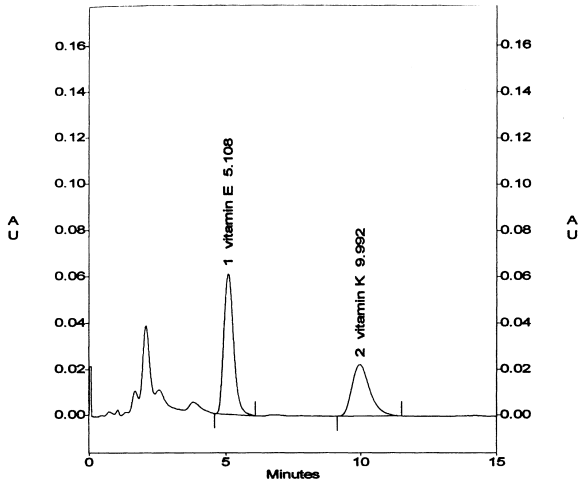

Fig. 1. Chromatogram of the working standard of vitamin E and vitamin K_1 (for details see Section 2).

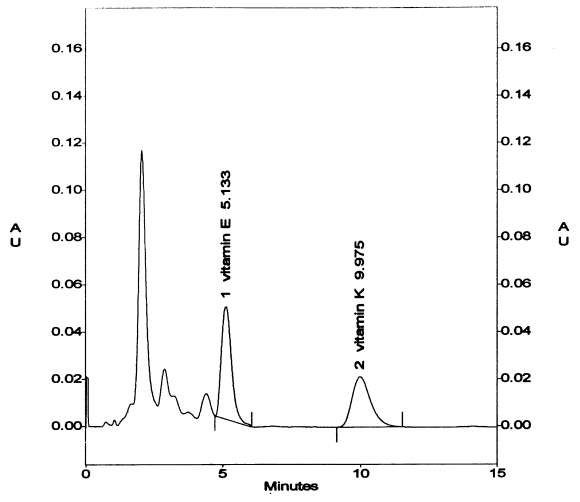

Fig. 2. Chromatogram of vitamin E in adipose tissue (for details see Section 2).

R.S.D. for the level of analyte in samples for both tissues, being higher in the inter-assay than in the intra-assay analysis, as would be expected.

Recoveries are close to 100%. It does not statistically differ from 100% for mammary gland (98±11%) and it differs but is acceptable in adipose tissue (95±3%).

So the assayed method is easy to perform as it needs only direct α-tocopherol extraction from samples, without tedious pretreatment, only a small amount of sample is used and validation parameters show that it is adequate for quantification.

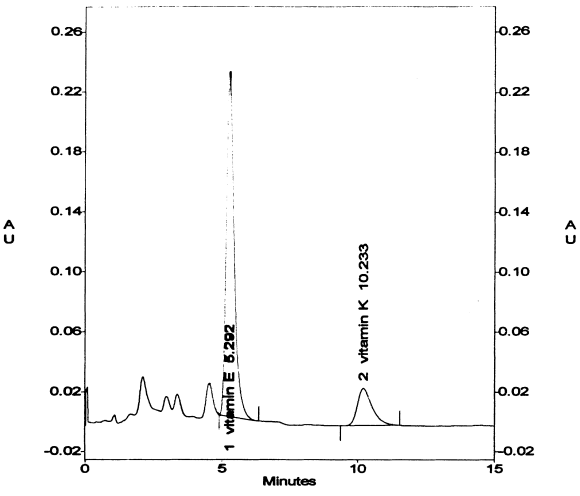

Fig. 3. Chromatogram of vitamin E in mammary gland (for details see Section 2).

Table 1
Validation parameters of vitamin E measurement in rat adipose tissue and mammary gland extracts

	Vitamin E	
	Adipose tissue	Mammary gland
Standard linearity		
Intercept	−0.003±0.03	0.05±0.2
Slope	0.87±0.02	0.16±0.02
r	0.99	0.99
Range (nmol/tube)	0.56–4.51	2.18–17.4
Sample linearity		
Intercept	−0.15±0.03	0.01±0.2
Slope	$0.0132\pm7\cdot10^{-5}$	$0.16\pm6\cdot10^{-4}$
r	0.999	0.99
Precision (μg/g tissue)		
Intra-assay		
Mean	57	234
R.S.D. (%)	4	3
Inter-assay		
Mean	53	205
R.S.D. (%)	9	15
Accuracy		
Recovery (%)	95	98
R.S.D. (%)	3	11

The following results were found in adipose and mammary gland tissues of virgin and pregnant rats 24 h after receiving one oral dose of vitamin E: vitamin E content in adipose tissue appeared very similar in virgin (33.35±14.5 μg/g) and pregnant rats (35.97±6.4 μg/g), values being in the range given by Bieri and Evarts [12] for tissues from untreated non pregnant Sprague Dawley rats, and higher than those found by Liu and Huang [14] in Long Evans rats. However, a statistically significant (Student test, $p<0.05$) higher vitamin E content was found in mammary gland from pregnant than virgin rats.

Although there are no previous reports on vitamin E content in mammary gland, present findings fit with the known increased activity of lipoprotein lipase in mammary gland of the late pregnant rat [13]. Since vitamin E is normally transported in blood associated to triglyceride-rich lipoproteins [15], and tissue uptake depends on lipoprotein lipase

activity [16], it has also been proposed that tissue vitamin E uptake depends on the same enzyme [17]. Present results therefore agree with this hypothesis, suggesting that the uptake of vitamin E in mammary gland is enhanced during late pregnancy. Throughout this mechanism, vitamin E from maternal diet becomes available to the suckling newborn.

The unchanged content of vitamin E in adipose tissue in pregnant rat versus virgin rats contrast with the decreased lipoprotein lipase activity known to be present in this tissue during late gestation [18,19], but we have recently found that the gut absorption of vitamin E is more efficient in pregnant than in virgin rats (unpublished results), as it was previously found to be the case for dietary triglycerides [20]. Higher circulating vitamin E levels in pregnant than in virgin rats would therefore compensate for the decreased lipoprotein lipase activity in adipose tissue of pregnant rats, allowing a similar vitamin E content in this tissue in both groups.

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