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A new HPLC method for the determination of tocopherol in rat plasma

Une nouvelle méthode de dosage du tocophérol dans le plasma de rat par chromatographie liquide à haute performance

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Abstract – Objective: The objective of the present work is the simplification of sample treatment for vitamin E measurements in rat plasma by high-performance liquid chromatography (HPLC), then the evaluation of the effect of cadmium treatment on the vitamin E level. **Methods:** A HPLC method to determine vitamin E was developed with direct extraction with n-hexane-dichloromethane. The dry residue was redissolved in methanol. The method employs a Supelco Discovery[®] C18 column and methanol/water (95:5, v/v) as the mobile phase. After being developed, the method was validated with a fluorescence detector. To examine the effects of exogenous cadmium on the plasma vitamin E concentrations, male Wistar rats (weighing 150 ± 20 g) were exposed for 48 h and 10 days to oral intake of 15 and 30 mg/L cadmium (as CdCl₂) and vitamin E (10 mg/Kg of diet) simultaneously. **Results:** This method makes sample treatment easier, especially when working with a large number of samples. It has proved to be selective, linear, accurate and precise. Intoxication with cadmium was followed by a significant decrease in plasma vitamin E concentrations. The lowest level was obtained in the 30 mg/L dosing group after 10 days of cadmium administration. **Conclusion:** We describe a rapid reversed-phase (RP) HPLC procedure for the determination of vitamin E in plasma. This method might be useful in routine assessment because it saves on solvents and chromatographic time. Interestingly, α -tocopherol may play an important role in preventing oxidative stress induced by cadmium exposure by scavenging free radicals.

Key words: HPLC, vitamin E, cadmium, plasma, rat

Résumé – Objectif : L'objectif du présent travail est la simplification du traitement de l'échantillon pour le dosage de la vitamine E par chromatographie en phase liquide à haute performance (CLHP) dans le plasma, puis, l'évaluation de l'effet du traitement au cadmium sur les concentrations de la vitamine E. **Méthodes :** Nous proposons dans ce travail de mettre au point et de valider une nouvelle méthode de dosage par CLHP de la vitamine E dans le plasma. Après extraction liquide-liquide (n-hexane-dichlorométhane), la séparation est faite par CLHP avec détection à fluorescence en utilisant une colonne Supelco[®] C18 et une phase mobile composée de méthanol et d'eau (95:5, v/v). Pour examiner les effets de l'exposition au cadmium sur les concentrations plasmatiques de la vitamine E, des rats (150 ± 20 g) ont été exposés pendant 48 h et 10 jours à une prise orale de cadmium (CdCl₂, 15 et 30 mg/L) et de vitamine E (10 mg/kg d'aliment) simultanément. **Résultats :** Cette méthode rend le traitement des échantillons plus facile, en particulier lorsqu'on travaille avec un grand nombre d'échantillons. Il s'est avéré être simple, sensible et rapide pouvant être appliqué en routine. L'intoxication au cadmium a été suivie d'une diminution significative des concentrations de vitamine E, le taux le plus bas a été obtenu après 10 jours d'exposition à la dose de 30 mg/L de cadmium. **Conclusion :** Nous avons développé une méthode CLHP qui présente de nombreux avantages : une préparation des échantillons de durée relativement courte, simple et surtout peu coûteuse, ce qui rend cette méthode prometteuse pour des dosages routiniers de la vitamine E plasmatique. Fait intéressant, l' α -tocophérol peut jouer un rôle important dans la prévention du stress oxydatif induit suite à l'exposition au cadmium en piégeant les radicaux libres.

Mots clés : CLHP, vitamine E, cadmium, plasma, rat

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1 Introduction

Cadmium (Cd) is a very toxic heavy metal and an important environmental pollutant that is present in soil, air, water and cigarette smoke, and even in food. Cd is listed as one of 126 prior environmental pollutants. Cd has an extremely long biological half-life (10–30 years) that essentially makes it a cumulative toxin [1]. After intake and resorption, Cd enters the blood, where it binds to the erythrocyte membranes and plasma albumin [2]. In blood and tissues, Cd stimulates the formation of ROS, thus causing oxidative damage in erythrocytes and in various tissues, which results in a loss of membrane functions [3]. The oxidative stress caused by the net production of these free radicals has been associated with the development of different pathological conditions [4]. Protection against these acute actions of Cd can be achieved through the antioxidant systems [3, 5]. Free radical scavengers and antioxidants are useful for protection from Cd toxicity [6]. The increasing interest in the distribution of certain antioxidant vitamins, including vitamin E, arises from their protective role against free radicals. The beneficial effects of these vitamins in protecting from or even preventing those pathological conditions have already been established ([7, 8]). Vitamin E is widely studied as an antioxidant molecule and several authors have proposed that vitamin E supplementation could be useful in disturbances that are associated with oxidative stress. Vitamin E is a potent lipid-soluble antioxidant in the biological system that has the ability to quench free radicals directly and function as a membrane stabilizer. The protective effect of vitamin E supplementation against Cd-induced oxidative stress has been reported [5, 9–11]. Therefore, further studies are required to clarify the role of this compound, and it is essential to have simple and rapid methods available for the measurement of these antioxidants in a routine manner. Many methods have been reported for the determination of vitamin E in serum or plasma, because it is present in high enough concentrations that it can be detected easily. Liquid chromatography linked to fluorescence detection has been extensively used for vitamin E confirmatory analysis [12].

The first objective of the present work is the simplification of sample treatment for vitamin E measurements in rat plasma by HPLC and validation of the conditions with a fluorescence detector, then the evaluation of the effect of acute cadmium treatment on the vitamin E level in rats.

2 Materials and methods

2.1 Instrumentation

A Thermo Finnigan HPLC system equipped with a pump (model Spectra SYSTEM MP 1000XR), a degasser (model Spectra SYSTEM SCM1000) and an injector van (model Rheodyne 7125) with a 20- μ L sample loop were used. A fluorimetric detector (model Spectra SYSTEM FL3000) was also used. The chromatographic analysis was performed on a 5- μ m particle Supelco Discovery[®] C18 column (15 cm \times 0.46 cm) with a precolumn (1 cm \times 0.46 cm). The piloting of the HPLC system and the acquisition of results were executed with a HP computer, model KAYAK, with the chromatography software AZUR.

2.2 Reagents

All solvents were HPLC-grade quality. Hexane, ethanol and methanol were from Panreac. Dichloromethane (DCM) was from Janssen Chimica and D- α -tocopherol (C₂₉H₅₀O₂) 99% was from Acros Organics. Cadmium chloride was obtained from Sigma.

2.3 Animals and plasma sampling

Adult Wistar male rats (SIPHAT, Tunisia), weighing 130–170 g at the beginning of the experiment, were randomly divided into the following groups: control rats and Cd-exposed rats (CdCl₂, 15 or 30 mg/L, in drinking water). Animals were housed in groups of six in cages at 25 °C, under a 12:12 h light/dark cycle, with free access to food and water. Animals were cared for under the Tunisian code of practice for the Care and Use of Animals for Scientific Purposes. The experimental protocols were approved by the Faculty Ethics Committee (*Faculté des Sciences de Bizerte*, Tunisia). Animals were sacrificed by decapitation after 48 h and 10 days of Cd administration. Blood was collected in EDTA-containing tubes and rapidly centrifuged to separate plasma. Plasma samples were stored in amber polypropylene tubes at –80 °C for a short period (1–2 weeks) before analysis.

2.4 Chromatographic analysis

The reverse-phase HPLC mobile phase was methanol/water (95:5, v/v) as the eluant, at a flow rate of 1 mL/min. Fluorescence was employed with excitation at 294 nm and emission at 330 nm.

2.5 Sample treatment

For the measurements of vitamin E, plasma (250 μ L) was deproteinized with ethanol (200 μ L) and vortexed for 2 min in an amber polypropylene tube. Fat-soluble vitamin E was then extracted into 1 mL of hexane/DCM (90:10) and the mixture was vortexed for 2 min. After centrifugation for 5 min at 3000 rpm at 4 °C, the lipophilic hexane phase was carefully transferred to another polypropylene tube and evaporated to dryness at room temperature under a stream of nitrogen. Then, the residue was reconstituted in 1 mL methanol, and 20 μ L of sample was injected into the HPLC system.

2.6 Validation

Individual stock solutions of commercial vitamin were prepared in methanol, and consisted of 10 mg/mL α -tocopherol. The vitamin E peak on the chromatograms was quantified by an external standard. The reproducibility of the retention time and the repeatability of the detector response were tested by analyzing six injections of standard solution of vitamin E (1 μ g/mL). Linearity standards were verified in each case by analyzing the triplicates containing 0.5, 0.8, 1, 2 and 5 μ g/mL

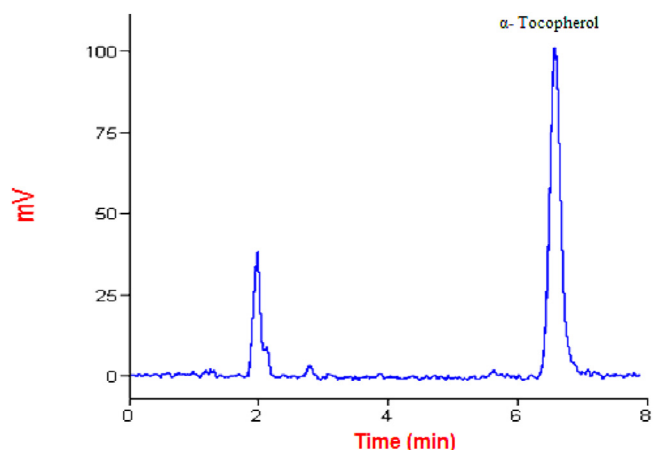


Fig. 1. Representative chromatogram of α -tocopherol standard solution (2 $\mu\text{g/mL}$) detected with fluorescence detector.

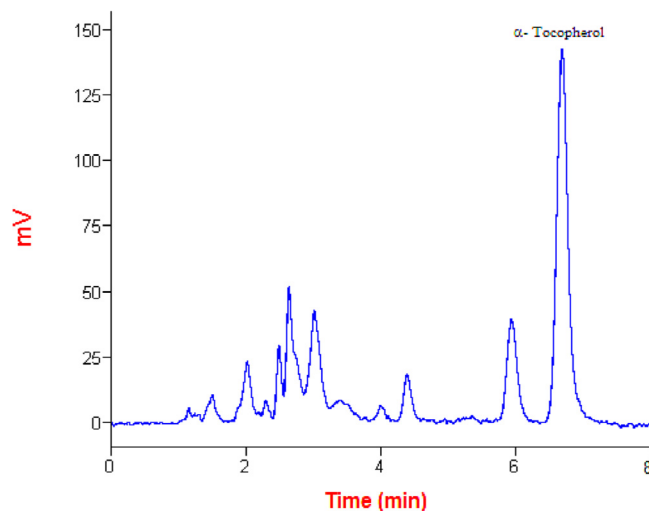


Fig. 2. Representative chromatogram of plasma sample detected with fluorescence detector.

of the vitamin E. Recovery was calculated through the linearity range by adding 1 and 4 $\mu\text{g/mL}$ of the vitamin E to 250 μL of plasma. The detection limit was determined by the analysis of 0.1 $\mu\text{g/mL}$ vitamin E standard solution.

2.7 Statistical analysis

Data were analyzed using Statistica Version 6.0. Results were expressed as means \pm SEM and differences between the controls and treated animals were determined by Student's *t*-test. The level of significance was set at $p < 0.05$.

3 Results and discussion

3.1 Results

This method requires a short time of analysis; only 8 min. Vitamin E was identified on the basis of the retention time when compared with standard solutions (6.65 min, RSD = 0.12%, $n = 6$). Figures 1 and 2, respectively, show the chromatographic profiles of α -tocopherol standard solution and the plasma sample. The reproducibility of the retention time and the repeatability of the detector response were good, with RSD respectively 0.12 and 3.69 for standard aliquots. The detection limit (signal-to-noise ratio of 3) obtained was 2 ng with a direct standard solution injection. Quantification was performed using a linear calibration curve established in the range of 0.5–5 $\mu\text{g/mL}$ with a correlation coefficient (r) of 0.998 (figure 3). The mean extraction recoveries are presented in Table I. α -tocopherol recovery was higher than 94%. The plasma vitamin E concentration showed a linear decrease in all groups treated with cadmium, depending on the duration of exposure (48 h and 10 days) and Cd concentration (15 mg/L and 30 mg/L). The lowest level was obtained in the 30 mg/L dosing group after 10 days of Cd administration (Table II).

4 Discussion

Cadmium is an ubiquitous environmental contaminant that poses serious health risks. Cd has been shown to induce

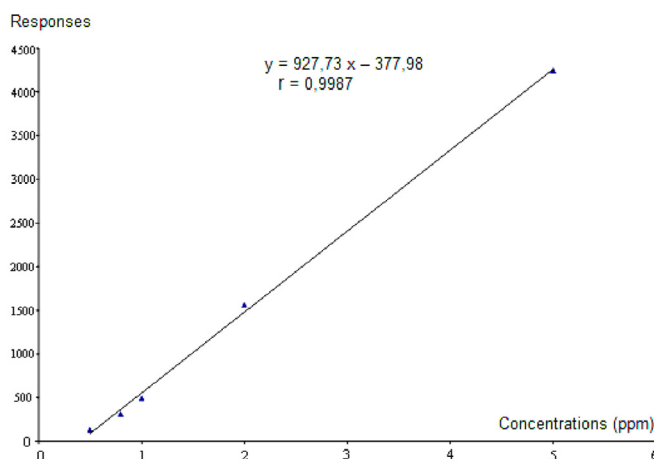


Fig. 3. The calibration curve of α -tocopherol.

Table I. Percentage of α -tocopherol recoveries from samples at different spiking levels.

	Spiking levels ($\mu\text{g/mL}$)	Recoveries (%) ($n = 3$)	RSD (%)
Plasma	1	94.24	1.84
Plasma	4	96.28	0.36

lipid peroxidation and cause excretion of lipid metabolite in urine [3]. Acute Cd-induced toxicity may be caused by the exhaustion of GSH stores and the increase in oxidative stress [3, 5]. Acute Cd exposure can increase oxidative stress by producing superoxide anions and nitric oxide [13]. Vitamin E, which is also designated as the first line of defense against oxidative stress, is an important antioxidant in biological systems because of its association with the cell membrane, and its ability to act directly on ROS and prevent lipid peroxidation [14]. α -tocopherol is the most active form of vitamin E and the most abundant lipid-soluble antioxidant in human plasma [15]. The ability of α -tocopherol to suppress lipid

Table II. Concentrations of plasma α -tocopherol in control rats and treated with cadmium.

Groups	Control		Treated with CdCl ₂		
	Time	Doses	48 h		10 days
15 mg/L			30 mg/L	15 mg/L	30 mg/L
D- α -tocopherol (μ mol/L)	12.86 \pm 0.24	11.94 \pm 0.19*	10.68 \pm 0.30**	11.03 \pm 0.19**	9.22 \pm 0.25**

Data represent the means \pm SEM of six animals per group

* $p < 0.05$, ** $p < 0.01$ compared to control

peroxidation is generally attributed to its scavenging of chain-carrying lipid peroxyl radicals.

The results of our experiments show that the concentrations of vitamin E in the plasma significantly decreased in Cd-treated rats in comparison with the control animals. Plasma levels of vitamin E also decreased in a time- and dose-dependent manner (7.15% and 16.90% after 48 h and 14.20% and 28.30% after 10 days of treatment with, respectively, the doses of 15 and 30 mg/L Cd). The decreased concentration of vitamin E in the plasma of Cd-treated animals could explain the protective role of vitamin E on Cd-induced oxidative stress. Vitamin E is not likely to bind Cd ions directly; therefore, its protective effect might happen due to its antioxidant property [9]. Vitamin E is capable of scavenging oxygen-derived free radicals as well as lipid peroxy radicals [16]. α -tocopherol reacts with peroxy radicals to form α -tocopheroxyl radicals, which are further oxidized into α -tocopheryl quinone. α -tocopheryl quinone is less reactive than the oxygen-containing radicals. Therefore, the chain reaction of free radical-induced membrane oxidation is interrupted, and the cell membrane integrity is protected. Vitamin E can also structurally stabilize biomembranes because of the physicochemical interactions between α -tocopherol and unsaturated fatty acids [17]. The membrane lipid-stabilizing effect of vitamin E can occur by stabilizing the lipid bilayer through a Van der Waals interaction of tocopherols with unsaturated fatty acids of phospholipids or by stabilizing the polypeptide chains of membrane proteins [18].

Antioxidants such as vitamin E are important for preventing the damage caused by cadmium-induced oxidative damage [19–22]. Pavlovic *et al.* [23] showed that vitamin E played a critical role in detoxifying Cd toxicity. Nemmiche *et al.* [24] suggest that cadmium induces an oxidation of cellular lipids and proteins, and also the administration of α -tocopherol can reduce Cd-induced oxidative stress and improve the glutathione level together with other biochemical parameters. Moreover, Pari and Murugavel [25] found that the levels of plasma lipid peroxidation markers, thiobarbituric acid reactive substances and lipid hydroperoxides, significantly increased while the levels of plasma reduced glutathione (GSH), and vitamins C and E significantly decreased in Cd-administered rats (3 mg/kg for 3 weeks). In agreement with this, the depletion of antioxidant vitamin E levels in plasma may be caused by increasing utilization to reduce Cd-induced oxidative stress.

5 Conclusions

In this study, we describe a rapid reversed-phase (RP) HPLC procedure for the determination of vitamin E in plasma.

This method might be useful in routine assessment because it saves on solvents and chromatographic time. Nevertheless, selectivity is usually provided by the chromatographic system and the sensitivity is good enough for α -tocopherol, the most abundant form of vitamin E in plasma. A fluorescence detector is the best option for tocopherol analysis due to its sensitivity, selectivity and easy handling. Interestingly, α -tocopherol may play an important role in preventing oxidative stress induced by cadmium exposure, as an exogenous antioxidant, scavenging free radicals and superoxide.

Conflicts of interest. The authors declare that there are no conflicts of interest.

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