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Short communication

Sensitive method for measuring tissue α-tocopherol and α-tocopheryloxybutyric acid by high-performance liquid chromatography with fluorometric detection

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Abstract

The nonhydrolysable tocopherol ether analog, *d*-α-tocopheryloxybutyric acid (TSE), and its tocopherol ester counterpart, *d*-α-tocopheryl hemisuccinate (TS), have been shown to possess anti-tumor activity. In the present study, a sensitive high-performance liquid chromatography (HPLC) method using fluorometric detection is described for the simultaneous determination of TSE and α-T in biological specimens. Maximal sensitivity for the measurement of TSE and α-T was observed with the wavelengths, 210 nm excitation and 300 nm emission. Using an internal standard (I.S.) method, the amount of these tocopherol compounds was determined in standards, liver homogenates isolated from rats administered TSE–tris salt or vehicle (saline) and in HL-60 human leukaemia cells incubated with TSE–tris salt or saline. Treatment with TSE resulted in the significant accumulation of TSE, but not α-T, in the liver and HL-60 cells. © 1998 Elsevier Science B.V.

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1. Introduction

Numerous studies attest to the anti-tumor activity of the vitamin E ester, TS in a wide variety of tumor cell lines [1–3]. The mechanism(s) for the observed anti-tumor actions of TS remains unclear. One hypothesis is that the anti-tumor effects of TS result from the action of α-T released by cellular esterases. However the in vitro administration of α-T did not result in significant anti-tumor activity [2,3]. These findings suggest that TS possesses unique anti-tumor actions that are not dependent on the cellular release of α-T but instead are related to the intact TS molecule. We have recently synthesized the ether analog of TS, TSE [3]. Unlike TS, TSE does not release α-T following base hydrolysis [3] and is not expected to increase cellular α-T levels. Using leukemia and breast tumor cell lines, we have demonstrated that TSE and TS possess similar anti-tumor activity, again suggesting that the intact TSE and TS molecules are the active tumoricidal species [3,4]. Unfortunately the absence of a sensitive method to measure TSE in biological systems continues to limit our conclusions from these studies since the cellular accumulation of TSE and α-T following TSE administration have not been determined. In the

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present study, we investigated whether a method using HPLC with fluorometric detection could be adapted for the simultaneous measurement of TSE and α-T in biological samples.

2. Experimental

2.1. Chemicals and standard solutions

Ascorbic acid, HPLC grade methanol and hexane were obtained from Fisher Scientific (Pittsburgh, PA, USA). Absolute ethanol was obtained from J.T. Baker (Phillipsburg, NJ, USA). Sodium dodecyl sulfate (SDS) was obtained from Sigma (St. Louis, MO, USA), RPMI 1640 cell culture media and constituents from Gibco BRL (Grand Island, NY, USA) and heat-inactivated fetal bovine serum from Hyclone (Logan, UT, USA). The internal standard (I.S.), *d*-δ-tocopherol (δ-T) (96%) and α-T (96%) were a gift from Henkel (La Grange, IL, USA). *d*-α-Tocopheryloxybutyrate tris salt (TSE–tris) (95%) was synthesized according to the procedures reported by Fariss et al. [3]. Stock standard solutions were prepared by dissolving approximately 10 mg of standards in 10 ml of methanol. All standard stock solutions were prepared fresh prior to the determination of response factors for each tocopherol compound.

2.2. Animals and rat liver homogenization

Male Sprague-Dawley rats (175 to 225 g) were obtained from Simonsen Labs (Gilroy, CA, USA) and given food (Purina Rat Chow 5001) and water ad libitum in our animal facility for at least 3 days before use. TSE–tris was suspended in saline (30 mg/ml) with sonication and given i.p. at a dose of 0.19 mmol/kg body weight (approx. 120 mg/kg). In vehicle control rats, saline was given i.p. at a dose of 4 ml/kg body weight. The food was withdrawn immediately after TSE or vehicle administration and animals were sacrificed 18 h later. Animals were anaesthetized with diethyl ether and livers were removed for homogenization as previously described [5]. Proteins were measured according to the procedures of Lowry et al. [6] as modified by Peterson [7]. All procedures were approved by the Washington State University Animal Care and Use Committee and met or exceeded current local and federal regulations.

2.3. Tumor cell culture

The human promyelocytic leukaemic cell line HL-60, described by Collins et. al. [8], was maintained in RPMI 1640 media supplemented with 1% sodium pyruvate, nonessential amino acids, L-glutamine, penicillin, streptomycin and 10% heat-inactivated fetal bovine serum. Cultures were passed twice weekly and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Control cultures exhibited a doubling time of approximately 24 h. To determine the cellular concentration of TSE, HL-60 cells were suspended at a concentration of 4×10^5 cells per ml and treated with saline or TSE–tris in saline (50 m*M* final concentration). After incubation at 37°C for 3 h, an aliquot from each cell culture containing 2×10^6 cells was pelleted, washed twice with phosphate buffered saline and analyzed for α-T and TSE content.

2.4. Tocopherol determinations

α-T and TSE levels were measured according to the method of Fariss et al. [9] with slight modifications. Aliquots of the I.S., δ-T (1.6 nmol), were added to biological samples and standards in 1.9 ml microfuge tubes. Samples were resuspended with sonication in 0.3 ml of 1% ascorbate in 0.1 *M* SDS and 0.45 ml absolute ethanol. The sample mixture was then extracted once with hexane by adding 0.8 ml hexane and vortexing for 30 s. Hexane extracts were evaporated to dryness with nitrogen and then resuspended in 1 ml of methanol containing 2.5% ascorbate. Extracted and nonextracted standards of α-T and TSE (ranging from 0.05 to 5 nmol/sample) were run with each assay. The extraction solutions, 100 m*M* SDS–1% ascorbate and methanol–2.5% ascorbate were stored at 4°C until use and were made fresh monthly. Samples containing TSE tended to degrade if stored for prolonged periods of time in methanol–2.5% ascorbate at 4°C. Therefore, samples containing TSE were run within a week after being resuspended in methanol–2.5% ascorbate (less than 1% degradation during this time period). Portions

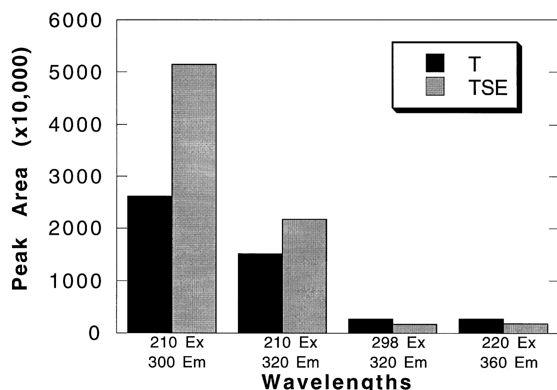

Fig. 1. Excitation and emission wavelengths for α-T (T) and TSE detection. The data presented are the area of peaks recorded after injecting 550 pmol of α-T or 550 pmol TSE at the indicated excitation (Ex) and emission wavelengths (Em) using the HPLC conditions described in the Section 2. Values are the mean of three separate HPLC runs with a S.D. of less than 5%.

(100 μl) of the methanol/ascorbate were analyzed on HPLC for the determination of α-T, δ-T and TSE. Quantitation of these compounds was based on the I.S. method.

2.5. HPLC determinations

Samples were injected on a Hewlett–Packard 1050 HPLC equipped with a McPherson 749 fluorescence detector and Spherisorb ODS II column (250×4.6 mm I.D., 5 μm; Alltech, Avondale, PA, USA). The mobile phase was 96% methanol which was run isocratically at a flow-rate of 1.8 ml/min. Excitation and emission wavelengths of 210 nm and 300 nm, respectively, were used for all tocopherol determinations unless otherwise noted. These wavelengths yielded maximal sensitivity for tocopherol determinations (see Fig. 1).

3. Results and discussion

Many different excitation and emission wavelengths, with varying sensitivities, have been used for the fluorometric detection of tocopherols by HPLC methods. Though α-T esters (e.g. TS and tocopheryl acetate) cannot be detected by fluorometric detection, it is not known whether tocopherol ethers exhibit fluorescence. Thus a series of excitation wavelengths and emission filters were tested to determine if TSE, a α-T ether analog, retained the fluorescence exhibited by unesterified α-T. As Fig. 1 indicates, the TSE molecule is measurable with fluorometric detection. Maximal sensitivity was obtained for both TSE and α-T with an excitation wavelength of 210 nm and an emission wavelength of 300 nm. These settings were used for all subsequent measurements of tocopherol compounds. At these settings, the peak areas of TSE and α-T yielded a linear response (r=0.9975) over the range of 6–3800 pmol injected. The limits of detections for both TSE and α-T were about 3 pmol injected. This value compares to a detection limit for α-T of 0.1 pmol injected using electrochemical detection [10] and 110 pmol injected using UV detection [11]. Since TSE cannot be measured with an electrochemical detector (due to its limited redox potential), fluorometric detection appears to be the most practical and sensitive method commonly available for detection of TSE with HPLC.

A single hexane extraction of standards recovered about 60% of the total TSE and α-T. A similar extraction efficiency was also observed for the I.S., δ-T. Peak areas for TSE and α-T were linear (r= 0.9942) over the range of 5–1000 pmol injected following extraction with hexane. Using this extraction procedure and the I.S. method, values obtained for TSE and α-T standards (5 to 500 pmol injected) were within 90% of predicted values. Aliquots of TSE (3500 and 50 pmol) were also added to liver homogenates from untreated rats. Subsequent extractions and analysis yielded TSE values which were within 90% of predicted values.

In a representative chromatogram of δ-T (I.S.), α-T, and TSE standards, these compounds had retention times of 8.0, 11.5 and 13.4 min respectively. When TSE was extracted from biological samples from a variety of sources, no interfering peaks were observed using the 210 nm excitation/300 nm emission wavelengths (data not shown).

Rats injected with 0.19 mmol/kg TSE–tris or saline (vehicle) were sacrificed 18 h later with their livers removed for homogenization. TSE levels in rat liver homogenates (0.65±0.19 nmol/mg protein, n= 3) were about five fold higher than endogenous α-T levels. No increase was seen in α-T levels in rat liver

homogenates following TSE administration. In saline treated rats, α-T levels were measured at 0.15 [±0.02 nmol/mg protein (n=4)], while in TSE-treated animals α-T values were 0.13 [±0.01 nmol/mg protein (n=3)]. These results indicate that in contrast to TS [12], TSE is not converted to free α-T in any appreciable amount in rat liver and consequently TSE appears to be resistant to hydrolysis by hepatic esterases.

The cellular uptake of TSE (2.43 nmol/10^6 cells) was also observed in HL-60 human leukemia cells following a 3-h incubation with a cytotoxic dose (50 μM) of TSE. The absence of any measurable α-T in these tumor cells following incubation with TSE indicates that TSE is not hydrolyzed by this tumor cell line. These results suggest that both normal and tumor cells have the capacity to take up large amounts of TSE from the extracelluar space but cannot convert cellular TSE to α-T.

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