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Note

Improved assay for α-tocopherol in the picogram range, using gas chromatography—mass spectrometry

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Vitamin E is the generic name for the biologically active members of the fat-soluble tocopherol and tocotrienol families; α-tocopherol is the most biologically active form. Two theories of the role of vitamin E in the lung have been promoted. According to the antioxidant theory, vitamin E acts as a lipid antioxidant by inhibiting the free-radical-mediated peroxidation of unsaturated lipids by acting as a free-radical scavenger [1, 2]. Other investigators, however, disagree with the primacy of an antioxidant role and suggest a specific metabolic role for vitamin E [3, 4].

Three factors were paramount in our resolve to develop a simple, direct, quantitative microtechnique for determining vitamin E in lung tissue. First, we recognized some years ago from our structural studies [5—7] that the lung does not react homogeneously to oxidant gases. Therefore, meaningful results from the analysis of the whole tissue could be masked by including large areas of the lung that appeared unaffected. Second, vitamin E is very sensitive, and is oxidized readily when exposed to air or other oxidizing agents. Thus extreme care must be exercised to obtain accurate data from tissue samples subjected to complicated extraction procedures. Third, a major deficiency in studies of vitamin E in the lung has been that the tissue levels of vitamin E were rarely determined quantitatively; the actual amount in the lung was presumed to be proportional to the amount fed to the animals.

To overcome the above difficulties we developed a procedure that uses freeze-dried cryostat sections, as well as microdissections of similar freeze-dried sections, as source materials for vitamin E analyses with gas chromatography—mass spectrometry (GC—MS). This procedure is direct, quantitative, and reproducible. Analyses can be conducted on a very small amount of dry tissue; thus the procedure permits us to analyze individually tissues such as

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the airways, alveolar parenchyma, focal areas of response (juncture of the terminal bronchiole and proximal alveolar duct region), blood vessels, and connective tissue obtained by microdissection of freeze-dried sections of the lung.

Until now, available methods for α-tocopherol determination (refs. 8—10 and references cited therein) have lacked the sensitivity and specificity needed in our research, and all methods require multi-step extraction—purification procedures that are not only time-consuming but cause significant losses of this very labile compound, particularly when working with sub-nanogram quantities. Major features of our method include: (1) A stable-isotope labeled α-tocopherol, used as an internal standard. (2) Increased stability of standards and endogenous tocopherols by working exclusively with their trimethylsilyl (TMS) derivatives. (3) A rapid extraction procedure that requires no further purification before the GC—MS analysis. (4) The possibility of analyzing other tocopherols simultaneously by monitoring their molecular ions. (5) The precise selection of any tissue or area thereof, as well as blood, may be used as source material without any modification of the procedure.

EXPERIMENTAL

Materials

The silylation reagent [bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane], and the solvent (pyridine) were obtained from Regis Chemical (Chicago, IL, U.S.A.). The GC column packing (3% Silar-10C on 100—120 mesh Gas-Chrom Q) and the cholestane standard were obtained from Applied Science Labs. (State College, PA, U.S.A.). The α-tocopherol standard was obtained from Sigma (St. Louis, MO, U.S.A.).

The deuterated α-tocopherol standard was prepared by reducing α-tocotrienol (from Hoffmann-LaRoche, Basel, Switzerland) with deuterium at room temperature and pressure in ethanol-*d*, using Pd/C catalyst. The product, a mixture of deuterated α-tocopherols resulting from both hydrogenation and exchange, was purified by thin-layer chromatography (TLC) on silica gel using cyclohexane—diethylether (4:1, v/v) as the solvent. The d_{13} species was the most abundant of the many labeled variants in this mixture.

Instrumentation

Analyses were performed on an LKB 9000 combination gas chromatograph—mass spectrometer equipped with a data system [11] based on a DEC PDP-12 computer. Masses 502 [molecular ion of tocopherol trimethylsilyl (TMS) ether] and 515 (molecular ion of tocopherol-d_{13} TMS ether) were monitored at 20 eV with a computer-controlled variable accelerating voltage system [12]. GC was performed with a 2 m × 2 mm glass column of 3% Silar-10C on 100—120 mesh Gas-Chrom Q at 230°C, with an injector temperature of 300°C and a helium carrier gas flow-rate of 30 ml min^{-1}.

Tocopherol standard solutions were assayed on a Hewlett-Packard Model 5710A gas chromatograph with a flame ionization detector (FID). The column had the specifications given above, but was operated at 190°C.

Tissue preparation

Blocks of fresh tissue, approximately 5 mm^3 each, were obtained from each lung to be studied. These were immersed in liquid nitrogen and stored at −80°C. The blocks were mounted on a cryomicrotome chuck, trimmed, and sectioned at 10 to 50 μm, at −20°C. The sections were freeze-dried and then weighed on a microbalance (accurate to 0.1 μg). To determine tissue-specific levels of the vitamin, specific tissues (e.g. airway, alveolar parenchyma, vascular, and connective tissue) were isolated by microdissecting the freeze-dried sections under a stereomicroscope. When necessary, homologous micro-sections were pooled before weighing.

Extraction

Dry samples weighing between 5 and 50 μg were placed in a 0.5-dram vial equipped with a PTFE-lined screw cap. Silylating reagent (15 μl), 15 μl of pyridine, and approximately 40 ng of deuterated α-tocopherol in 2 μl of silylation reagent—pyridine were added, and the vial was capped tightly. After at least 10 min at room temperature, extraction and derivatization were complete. Then the sample was injected, in 2-μl aliquots, directly into the GC—MS system for analysis.

Calibration

Mixtures of known weights of α-tocopherol and deuterated α-tocopherol (both from stock solutions in silylation reagent—pyridine, i.e. TMS-derivatized) were prepared and analyzed. A calibration curve was prepared by plotting the ratio of peak areas in the MS response to α-tocopherol and its deuterated analogue as a function of the weight ratio of these compounds in each sample mixture. The equation for this curve was determined by linear regression analysis.

Standards

Stock solutions (10 mg ml^{-1}) of α-tocopherol (for calibration) and deuterated α-tocopherol (for the internal standard) were maintained in silylating reagent—pyridine (1:1). Before each use, these were assayed by GC with FID by combining an aliquot with a cholestane solution of known concentration. Cholestane was selected as a primary standard because of its known purity, stability, and suitable GC retention time. A relative molar response ratio of 0.99 was determined for α-tocopherol TMS ether versus cholestane, using the labeled tocopherol which was purified by TLC and shown by GC to be 96% pure. The concentration of an aliquot of the labeled stock solution was verified (re-established if necessary), and then the aliquot was diluted to approximately 20 μg ml^{-1}, which was found to be a level suitable for an internal standard.

RESULTS AND DISCUSSION

The main difficulty with all analyses for the tocopherols is that the compounds are unstable. Reference solutions cannot be prepared, stored, transferred, or otherwise manipulated without substantial risk of decomposition.

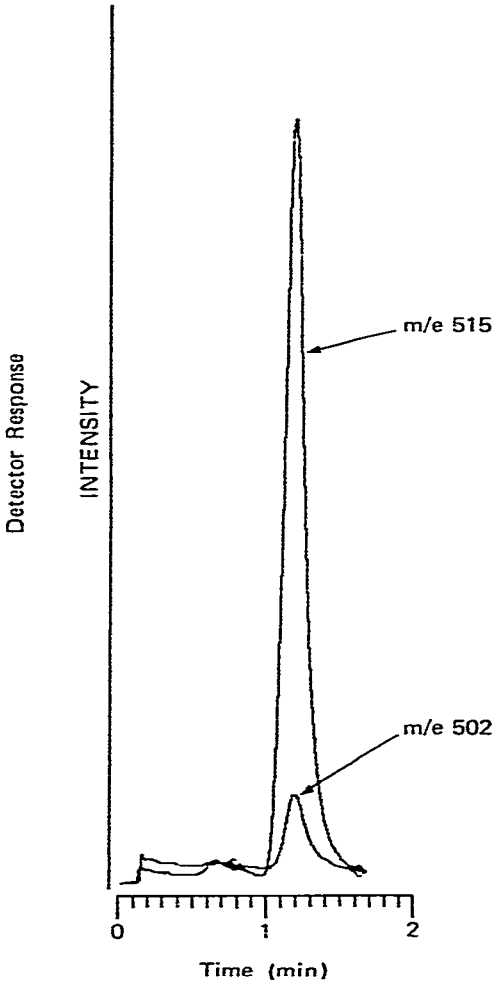

Fig. 1. GC—MS data from a 44-μg dry weight tissue section of rat lung. The *m/e* 502 trace represents 170 pg of α-tocopherol as its TMS derivative. The *m/e* 515 trace is the deuterium-labeled α-tocopherol TMS ether standard.

Likewise, vitamin E cannot be extracted from tissue or other substrates without risking significant, and often variable, losses of the vitamin.

To reduce these problems, we had previously added an internal standard (α-tocopherol labeled with deuterium) to the lung tissue before extraction. A chemically equivalent internal standard is used primarily because losses of the endogenous material should be compensated for by equivalent losses of the standard. However, one point which may be critical in the case of such labile compounds is seldom considered: the standard is added in solution form to a tissue (or blood) sample, while the endogenous material is within a cellular matrix, a distinctly different chemical and physical environment. The two compounds may decompose or be lost at different rates during extraction, particularly with multi-step, harsh extraction procedures. The variability in our early results was evidence of this problem. It soon became apparent that

TABLE I

α-TOCOPHEROL CONTENT OF LUNG TISSUE FROM ONE ADULT CONTROL RAT ON A NORMAL MAINTENANCE DIET

Each number is from a separate tissue section, microdissection, or pooled homologous microdissections.

Whole tissue section (μg/g dry weight)	Parenchyma	Connective tissue	Blood vessel walls	Airway
122	96	162	67	23
144	93	166	65	21
153	110	158	60	
	113			

we needed methods to stabilize both the endogenous and standard tocopherols, to evaluate routinely changes in concentration of our standard solutions, and to reduce the harshness of the extraction procedure while maximizing recovery yields.

Instability of the tocopherols may be significantly reduced by chemically blocking the free hydroxyl function. We have chosen to use a TMS derivative because it is easy to form and has excellent GC properties. All standard solutions are prepared as the TMS derivative, and their concentration may be routinely monitored by conventional GC methods versus a stable primary standard (e.g. cholestane). Special care must be taken, however, that the GC response is linear over the concentration range used, to avoid selective loss of either the tocopherol or cholestane in the GC system. This can be checked by injecting several different volumes and verifying peak ratio consistency.

Complications associated with conventional extraction procedures could be eliminated by changing the method of tissue preparation. For example, previous work [13] on measuring myoinositol in single cells showed the value of direct analysis of tissue constituents, using GC—MS ion monitoring and techniques of quantitative histochemistry. We believed that similar methods could be successfully applied to our lung research if enough vitamin E was present relative to potential interfering substances. Thus using weighed freeze-dried tissue sections or microdissections has several advantages. The departure from standard analyses of tissue homogenates allows, for the first time, the study of vitamin E in specific types of tissue within an organ. Also, of great significance is the fact that there is no need for any laboratory extraction or purification procedures. Extraction is accomplished by adding a silylating reagent directly to the dried tissue, together with the internal standard. Extraction is complete within a few minutes at room temperature. Furthermore, the vitamin E is converted rapidly in this process to its stable TMS derivative, which minimizes losses by decomposition.

Fig. 1 shows a typical chromatogram from the analysis of one tissue section weighing 44 μg (10% of the total extract was injected). This was ob-

tained from a rat that had been maintained on a diet deficient in vitamin E. The small peak (*m/e* 502) is due to endogenous α-tocopherol as the TMS ether, 170 pg in this aliquot, and indicates the excellent sensitivity of the technique in the picogram range. The large peak (*m/e* 515) is from the internal standard, α-tocopherol-d_{13} TMS ether. (Details of how the GC—MS technique of ion monitoring can be applied to similar assays using labeled internal standards have been thoroughly described [13, 14].) To emphasize the specificity of this method, one should note that a very large quantity of cholesterol (TMS ether) coelutes with the α-tocopherol TMS ether, but does not contribute any signal at the mass values chosen for this assay.

The ratio of peak areas observed in Fig. 1 is converted to a weight ratio of endogenous α-tocopherol to internal standard with a calibration curve prepared from known mixtures. A typical calibration had the equation $y = 8.098x + 0.028$ with a correlation coefficient of 0.9998, where y is the peak area ratio (*m/e* 502:*m/e* 515) and x is the weight ratio (α-tocopherol:α-tocopherol-d_{13}). Because there is no contribution at *m/e* 515 from unlabeled α-tocopherol TMS ether alone, the curve is linear [14]. The small positive y intercept results from a trace of unlabeled α-tocopherol in the internal standard, and a slope of considerably greater than 1 results because the d_{13} species chosen is only one of several deuterated variants of α-tocopherol in the standard. The calibration may be extended for other ranges of tocopherol concentration, but it is best to adjust the amount of internal standard added to the sample according to the anticipated endogenous α-tocopherol to obtain peak area ratios close to 1.

Table I presents some preliminary data resulting from the application of these methods to study α-tocopherol distribution in five different types of tissue in rat lung obtained through microdissection. Of special interest is the very remarkable difference in α-tocopherol content from one structural area to another; it is particularly low in the walls of both airways and blood vessels.

Much biochemical research on lung tissue exposed to oxidant gases is hampered because the lung tissue does not react homogeneously to the oxidant. The direct tissue analysis technique, using microdissection, will permit analysis both of the individual foci of injury at selected times during oxidant gas exposure, and of sections of airways, blood vessels, peripheral parenchyma, and even the epithelial layer of the upper airways. This selectivity gives us a means of studying the dynamics of vitamin E in specific lung locations during exposure to oxidant gases.

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