Routine assay for determination of α-tocopherol in liver

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Summary A rapid and accurate procedure for determination of α-tocopherol in liver has been developed. Extracts of saponified liver homogenates were purified by a simple and efficient chromatography step on Kieselgel. α-Tocopherol content of the purified extracts was determined by an automated gas–liquid chromatographic assay of the trimethylsilyl derivatives. Mean recovery for α-tocopherol added in vitro was 95.4 ± 1.2% (SEM, 149 estimations). The lower assay limit was ca. 0.5 μg α-tocopherol/g liver. An experienced analyst was able to process at least 12 chick, rat, or guinea pig livers per day.—**Kormann, A. W.** Routine assay for determination of α-tocopherol in liver. *J. Lipid Res.* 1980. **21:** 780–783.

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Evaluation of the biological activity of vitamin E products by chick liver storage assays requires α-tocopherol analysis in a large number of liver samples (1). Published methods for liver tocopherol determination (1–9) involve time-consuming steps which make them less suitable for routine assays. Consequently a reliable and efficient analytical procedure had to be developed. The present communication describes a method which allows accurate analysis of α-tocopherol in at least 12 liver samples per day by one analyst. Key steps are a rapid purification of liver extracts by Kieselgel chromatography and application of continuously operating automated gas–liquid chromatography (GLC) equipment.

Abbreviations: GLC, gas–liquid chromatography; HPLC, high pressure (performance) liquid chromatography; SD, standard deviation; SEM, standard error of the mean; TLC, thin-layer chromatography.

[1] With technical assistance of George Riss and Alain Rippstein.

MATERIALS AND METHODS

Materials

All-rac-α-tocopherol and *all-rac*-α-tocopheryl acetate were supplied by the Department of Quality Control, Hoffmann-LaRoche, Basle, Switzerland. The purity of these compounds was established according to the requirements of NF XIV which include tests by gas–liquid chromatography (purity of our samples > 97%), thin-layer chromatography, UV absorption, and heavy metal determination. Reagents and solvents of analytical grade were purchased from Merck, Darmstadt, Germany and used without further treatment.

Isolation of α-tocopherol from liver

The following procedures were carried out in rooms with filtered light (negligible UV emission) and with nitrogen flushing. Two g of liver were minced and homogenized (Polytron PTA 10-35, Kinematica, Lucerne, Switzerland) in 18 ml of 1.5% methanolic L-ascorbic acid solution. The homogenate was refluxed at 70°C for 5 min, 1.5 ml of 50% aqueous potassium hydroxide was added, and the samples were saponified at 70°C for 20 min. Twenty ml of 50% aqueous methanol were added to the cooled suspension prior to extraction with 50 ml of diethyl ether–cyclohexane 1:1. The methanol–water phase was discarded. The solvent phase was washed with 20 ml of 5% sulfuric acid and dried over anhydrous sodium sulfate. Solvent was evaporated under reduced pressure and the residue was dissolved in 5 ml of 5% ethyl acetate in n-hexane.

Purification of liver extracts

Liver extracts were purified by chromatography on Kieselgel 60, particle size 0.063–0.20 mm (Merck). Five g of Kieselgel were poured into glass columns (inner diameter 10 mm, length about 150 mm) with a spherical solvent reservoir at the top. The reservoir was large enough to hold the total volume of elution solvent (about 50 ml). Five ml of liver extract was pipetted on to the prewashed gel (2% ethyl acetate in n-hexane) and allowed to enter the gel. The sample was washed in with 5 ml plus 20 ml of the same solvent, and the eluate was discarded. α-Tocopherol was then eluted with 30 ml of 5% ethyl acetate in n-hexane. Elution proceeded without supervision and columns were allowed to run dry. Solvent was evaporated under reduced pressure and nitrogen, then the residue was dissolved in 1.0 ml of bis(trimethylsilyl)-trifluoroacetamide–pyridine 1:1 which contained 40 μg of stigmasterol (internal standard). Pyridine in the silylation mix could be replaced by an equal volume of tetrahydrofuran.

GLC determination of silylated α-tocopherol

Samples were analyzed with a Perkin-Elmer 900 gas chromatograph equipped with FID detector and an autosampler Hewlett-Packard 7076A. The glass column had a length of 1 m, ϕ_i 2.2 mm, and a stationary phase of 5% Apiezon L on Gas-Chrom Q 80–100 mesh (Applied Science). Operating temperatures were 290°C for the injection port and 260°C for the column. Carrier gas flow was 40 ml of He/min. α-Tocopherol content of the samples was calculated by the internal standard technique with a Varian CDS 240-32 computer. Each sample was analyzed in duplicate (1 μl/injection). Prior to their first use, columns were conditioned by repeated injections of α-tocopherol standard solutions until retention times and peak forms remained stable.

RESULTS AND DISCUSSION

Efficiency of the assay procedure

The primary aim of this investigation was to develop an efficient and accurate method for α-tocopherol determination in liver. The main improvement over existing procedures was accomplished by replacing time-consuming purifications of liver extracts with a simple chromatographic step.

Fig. 1 shows a typical example of the GLC assay of a chick liver extract. It demonstrates the efficiency of the purification by Kieselgel chromatography; accurate α-tocopherol determination by GLC was impossible if this purification step was omitted. The impurities prevented quantitative determination of α-tocopherol, increased the time interval between injections, and necessitated frequent replacement of clogged GLC columns and injection ports. If purified extracts only were assayed, a GLC column could be used for about 200 consecutive determinations. It was thus possible to apply automated GLC equipment which permitted continuous analyses of α-tocopherol. Consequently an experienced analyst was able to process at least 12 liver samples per day as both the Kieselgel step (with simultaneous treatment of several samples) and the GLC assay required a minimum of supervision.

Recovery of added α-tocopherol. Recovery for α-tocopherol added to chick livers (30 μg/g liver) prior to saponification was 95.4 ± 1.2% (mean ± SEM, 149 determinations). This value represents the recovery rate of all chick liver assays done up to present.

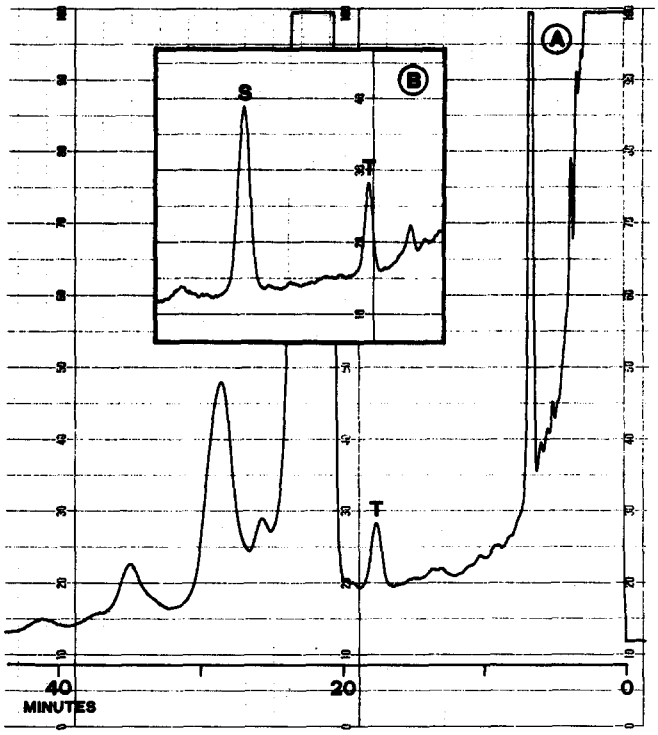

Fig. 1. Gas–liquid chromatograms of trimethylsilyl derivatives of α-tocopherol in a chick liver extract. Effect of purification of the extract by Kieselgel chromatography: (A) without purification, (B) after purification by Kieselgel chromatography. T, α-tocopherol; S, stigmasterol (internal standard). Only the central part of chromatogram (B) is shown, (A) and (B) are on the same scale. GLC conditions and Kieselgel chromatography procedures are described in "Materials and methods."

Mean recoveries for seven different sets of assays with at least nine determinations per set ranged from 90.1 to 103.1% (SEM ≦ 4.7%). Similar values were obtained with rat or guinea pig livers. All livers were from normally fed animals and contained small amounts of lipid. Analyses of fatty livers might yield lower recoveries due to less efficient α-tocopherol extraction in the presence of excess lipid (6, 10, 11).

Reproducibility of the GLC assay. Multiple GLC analyses of the same samples were utilized as an indicator of the reproducibility of the assay. In two typical examples, 16 analyses of a standard solution with 10.0 μg α-tocopherol/ml resulted in a standard deviation (SD) of 2.8%, and 10 analyses of a sample with 15.5 μg α-tocopherol/g liver yielded a SD of 4.5%. In addition, SD for duplicate GLC analyses were calculated (12). Typical examples demonstrate the excellent reproducibility of the assay: the SD was 1.5 to 1.7% for 10 different samples with 10.0 to 11.5 μg α-tocopherol/g liver, and the SD was 2.3 to 3.0% for 16 different liver samples containing 5.0 to 7.0 μg α-tocopherol/g.

The GLC apparatus could be operated continuously for several days without significant deterioration of performance; repeating duplicate analysis of any sample after 24 hr resulted in deviations of maximally 0.27 μg α-tocopherol/g liver. This represents 5.4% for a liver with 5.0 μg α-tocopherol/g and correspondingly less for livers with higher α-tocopherol concentrations.

Sensitivity of the assay. The lower assay limit of the whole procedure was about 0.5 μg α-tocopherol/g liver; it depended to some extent on the impurities still present in the extracts. Generally, it was possible to determine α-tocopherol contents of such depleted livers with an accuracy of 0.2 μg/g liver. This was sufficient for our purposes since the livers from our experimental animals contained at least 4 μg α-tocopherol/g. The sensitivity of the procedure can be increased by extraction of larger liver portions and/or concentrating the final solution for GLC analysis. The response of the FID detector was linear up to at least 100 ng of α-tocopherol injected.

Application of the method

More than 800 liver samples have been analyzed successfully according to the procedure described. It has been applied to α-tocopherol determination in chick, rat, and guinea pig liver in connection with liver storage assays of vitamin E products. Preliminary experiments indicated that the method is also suitable for α-tocopherol analysis of muscle. The GLC system as described may also be applied for determination of other tocopherols: analysis of mixed tocopherols (type 4-50, Distillation Products Industries, Rochester, NY) as silyl derivatives resulted in relative retention times of 1.00 for α-, 0.64 for β- + γ-, and 0.49 for δ-tocopherol, respectively. Slover, Lehmann, and Valis (13) reported very similar values.

Comparison to other assay methods

Additional experiments have been carried out to compare the GLC assay with alternative methods of α-tocopherol determination. Purified liver extracts have been analyzed with a high-performance liquid chromatography (HPLC) system. LiChrosorb Si60 (Merck) was the stationary phase, 5% ethyl acetate in n-hexane was the mobile phase; UV detection at 295 nm was used. This system was quite similar to others that have been published (14–16). Determination of α-tocopherol in 60 liver extracts with both the GLC assay and the HPLC system revealed that the latter requires more extensive purification of the extracts; interfering impurities caused erroneous values in about 15% of the samples. The GLC assay was much less sensitive to such impurities. An addi-

tional advantage of the GLC method was the increased stability of the trimethylsilyl ethers of α-tocopherol (stable for several days) in comparison to the samples with free α-tocopherol as used for the HPLC assay. This can be important if large numbers of samples have to be analyzed. HPLC determination of sufficiently clean liver extracts worked well and yielded no significant differences in comparison to the GLC assay. Recovery of added α-tocopherol was 94.9 ± 1.2% (mean ± SEM, 46 assays) for the HPLC system.

Fluorometric determinations of α-tocopherol according to the method of Taylor, Lamden, and Tappel (4) revealed a marked sensitivity of this assay system to interference by impurities in the liver extracts. This was verified by addition of known amounts of α-tocopherol to such extracts; in comparison to standard solutions, quenching of up to 50% of the expected fluorescence values was observed. Obviously the application of this technique requires more elaborate purifications prior to α-tocopherol determination than the simple Kieselgel step described above.

Similar problems were encountered with a combination of HPLC and fluorometric detection according to the system of Thompson and Hatina (16). In addition, these authors (16) reported that very frequent standardization of detector response was necessary to minimize experimental errors. Other workers (3, 6) described thin-layer chromatographic separation and quantitation of α-tocopherol in tissue extracts. But analyses of complex samples such as liver extracts cannot be done by simple one-dimensional techniques and require more time-consuming procedures (3, 6). Another drawback is the increased tendency of α-tocopherol oxidation on the TLC plate. Hess et al. (6) took advantage of this property and applied an 18-hr heating procedure with subsequent determination of the oxidation product(s) of α-tocopherol on the plate. It appears that in comparison to our GLC assay, TLC or paper chromatographic methods (8) offer no advantage with regard to efficiency or accuracy of analysis.

In summary, α-tocopherol determination by the GLC procedure was less sensitive to interference by impurities in liver extracts than detection by UV absorption or fluorescence. Consequently the latter techniques were less efficient as they required additional purification steps. The combination of saponification, extraction, chromatography, and GLC assay as described provides a reliable and rapid routine method for α-tocopherol determination in liver and probably also in other tissues.

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REFERENCES

1. Dicks, M. W., and L. D. Matterson. 1961. Chick liver-storage bioassay of alpha-tocopherol: methods. *J. Nutr.* **75:** 165–174.
2. Argrett, L. C., and C. R. Seward. 1968. An improved method for the quantitative determination of coenzyme Q and α-tocopherol in rat liver. *J. Assoc. Off. Anal. Chem.* **51:** 1236–1239.
3. Bieri, J. G. 1969. Chromatography of tocopherols. *In* Lipid Chromatographic Analysis. Vol. 2. G. V. Marinetti, editor. Marcel Dekker, New York. 459–478.
4. Taylor, S. L., M. P. Lamden, and A. L. Tappel. 1976. Sensitive fluorometric method for tissue tocopherol analysis. *Lipids.* **11:** 530–538.
5. Pesh-Imam, M., and R. O. Recknagel. 1977. Lipid peroxidation and the concept of anti oxygenic potential: vitamin E changes in acute experimental CCl_4-, $BrCCl_3$-, and ethanol-induced liver injury. *Toxicol. Appl. Pharmacol.* **42:** 463–475.
6. Hess, J. L., M. A. Pallansch, K. Harich, and G. E. Bunce. 1977. Quantitative determination of α-tocopherol on thin layers of silica gel. *Anal. Biochem.* **83:** 401–407.
7. Aftergood, L., and R. B. Alfin-Slater. 1978. Effect of administration of α- and γ-tocopherol on tissue distribution and red cell hemolysis in rats. *Internat. J. Vitam. Nutr. Res.* **48:** 32–37.
8. Bayfield, R. F., and L. F. Romalis. 1979. An improved method for the determination of α-tocopherol in sheep liver. *Anal. Biochem.* **97:** 264–268.
9. Machlin, L. J., J. Keating, J. Nelson, M. Brin, R. Filipski, and O. N. Miller. 1979. Availability of adipose tissue tocopherol in the guinea pig. *J. Nutr.* **109:** 105–109.
10. Nishiyama, J., E. C. Ellison, G. R. Mizuno, and J. R. Chipault. 1975. Micro-determination of α-tocopherol in tissue lipids. *J. Nutr. Sci. Vitam.* **21:** 355–361.
11. Bieri, J. G., S. L. Thorp, and T. J. Tolliver. 1978. Effect of dietary polyunsaturated fatty acids on tissue vitamin E status. *J. Nutr.* **108:** 392–398.
12. List, P. H., and L. Hörhammer, editors. 1971. Hagers Handbuch der Pharmazeutischen Praxis, volume 7. Springer, Berlin-Heidelberg-New York. 118–120.
13. Slover, H. T., J. Lehmann, and R. J. Valis. 1969. Vitamin E in foods: determination of tocols and tocotrienols. *J. Amer. Oil Chem. Soc.* **46:** 417–420.
14. Eriksson, T., and B. Sörensen. 1977. High-performance liquid chromatography of vitamin E. *Acta Pharm. Suec.* **14:** 475–484.
15. Nilsson, B., B. Johansson, L. Jansson, and L. Holmberg. 1978. Determination of plasma α-tocopherol by high-performance liquid chromatography. *J. Chromat.* **145:** 169–172.
16. Thompson, J. N., and G. Hatina. 1979. Determination of tocopherols and tocotrienols in foods and tissues by high performance liquid chromatography. *J. Liquid Chromat.* **2:** 327–344.