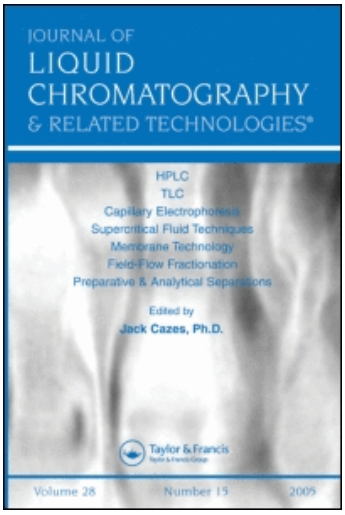

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DETERMINATION OF ALPHA-TOCOPHEROL LEVELS IN RAT MICROSOMES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A rapid, simple and sensitive method is described for the routine determination of alpha-tocopherol in microsomes. After a single extraction step with n-heptane extracts are injected onto a normal phase column using n-heptane/isopropanol (99.3/0.7, v/v) for elution; a fluorescence detector set at 215 nm excitation wavelenght and a cut-off filter emitting at 320 nm are used for detection. Total run time is less than 4 minutes.

INTRODUCTION

d-alpha-tocopherol (AT), a biological chain-breaking antioxidant, is considered to be the most effective free-radical scavenger of the naturally occurring forms of vitamin E (1). Therefore vitamin E levels in biological material are frequently assessed in terms of AT concentrations. Numerous methods for the determination of AT in food, pharmaceutical

products, and biological samples have been developed - frequently high performance liquid chromatography (HPLC) is applied (for review see 2,3).

However, none of the available HPLC methods for the determination of AT levels in microsomes appears to be sufficiently simple, selective or sensitive. Taylor et al. applied fluorimetry and HPLC with electrochemical detection following extraction with hexane (4). Murphy and Kehrer used reversed phase HPLC with electrochemical detection after extraction of microsomal suspensions with heptane (5). A detergent has been used to facilitate the extraction of AT from membrane-rich samples (6). Other methods involved saponification as a necessary step prior to extraction with hexane or heptane (7,8) and subsequent (reversed or normal phase) HPLC with fluorescence (7) or ultra-violet detection (8). Sterrenberg et al. (9) extracted microsomes directly with hexane and used the HPLC system of Rammell et al. (10): AT was eluted from a reversed-phase ODS column with a mixture of hexane and isopropanol.

For determination of AT levels in microsomes we report a fast, simple and sensitive method applying normal phase HPLC with fluorescence detection after a single extraction step with n-heptane.

MATERIALS AND METHODS

Chemicals and Materials

dl-alpha-tocopherol, ethanol p.a., and HPLC-grade isopropanol were purchased from Merck (Darmstadt, FRG). d-alpha-tocopherol-acetate (ATA) and 3,5-di-tert-butyl-4-hydrotoluene (BHT) were obtained from Sigma (St. Louis, MO, USA) and HPLC-grade n-heptane from Fisons (Loughborough, England). Nine male Wistar rats (300-350 g) were purchased from Winkelmann (Borchem, FRG). Microsomes from rat liver and brain were prepared in buffer (0.05 M Tris-HCl, 0.15 M KCl, pH 7.4) according to Burke and Orrenius (11) and stored at -70^{o}C. Microsomal protein levels were determined according to Lowry et al. (12).

Preparation of Samples

Aliquots of microsomal suspensions or buffer (1 ml) to which 0.1, 0.25, 0.5, 1.0, 1.5 or 2.0 µg AT was added, were mixed vigorously for 15 s with 1 ml of ethanol containing 50 µg/ml ATA as an internal standard and 500 µg/ml BHT for stabilization. n-Heptane (1 ml) was added and mixing was repeated for 15 s. After centrifugation for 5 min at 700 g the organic phase, containing AT as well as ATA, was used directly for HPLC.

High-Performance Liquid Chromatography

HPLC analyses was performed with a Kratos Spectroflow 400 pump and a Kratos Spectroflow 980 programmable fluorescence detector. A LiChrosorb Si-60-5 column (250x4,6

mm) and appropiate guard column (Chrompack, Middelburg, The Netherlands) were used. The excitation wavelength was set at 215 nm and a cut-off filter at 320 nm was used. Samples of 100 µl were injected with a Rheodyne 7125 sample injector. The solvent, n-heptane/isopropanol (99.3/0.7; v/v), was degassed prior to use in an ultrasonic bath and recycled at a flow-rate of 2.5 ml/min.

RESULTS AND DISCUSSION

Although some methods have been described for the determination of AT in microsomal suspensions (4-9) or biological samples (2,3), there is no standard procedure. Therefore several protocols with respect to stabilization, extraction, elution and detection were combined (5,8,10,13,14), resulting in the present report. The use of BHT as a stabilizing agent for AT and ATA was found necessary. Membrane reconstitution was avoided by using a polar phase of 50% ethanol/50% buffer which proved to be essential during the extraction procedure. By using normal-phase HPLC evaporation of the extracting solvent could be omitted. Fluorescence detection with low excitation wavelength of 215 nm showed sufficient selectivity and sensitivity while the total analysis time was kept short. Since this method yielded highly satisfactory results the use of detergents in the extraction procedure was not evaluated.

The retention times of AT and ATA were 2,8 and 1,7 min, respectively; their capacity ratios were 2.0 and 0.2, respectively. Both peaks were nicely separated and total analysis time was less than 4 min. There was no sign of interfering compounds co-eluting with the rapid appearing internal standard. Typical chromatograms are shown in Fig. 1. Spiking microsomal suspensions with extra AT prior to extraction resulted in an increase in peak-height on the AT location that was fully proportional to the extra amount of AT added. Calibration graphs, calculated from peak-height ratios, were linear over the range 0-2 µg AT/ml for both microsomes ($y = 0.47 + 0.22x$, $r = 0.9959$, n=3) and buffer ($y = 0.00 + 0.21x$, $r = 0.9987$, n=3). Since both calibration graphs were not different with respect to slope, correlation and coefficient of variation, data on the variability of the assay, presented in Table 1, were calculated from spiked buffer samples. The recovery of the internal standard was 103 ± 3% (mean ± S.D.). Limit of detection was at least 50 ng/ml or 5 ng absolute per injection.

This HPLC technique enables the fast and reliable determination of AT levels in microsomes. Measured peak-height ratios of AT versus ATA are compared with a calibration graph constructed from varying concentrations of AT in buffer; this method performed almost equally satisfactory however when the use of the internal standard was omitted.

In our laboratory this method has succesfully been used for the determination of AT concentrations in microsomes from rat liver and brain (Table 2). Tissue levels of AT were in agreement with data reported by others (4-9,15,16).

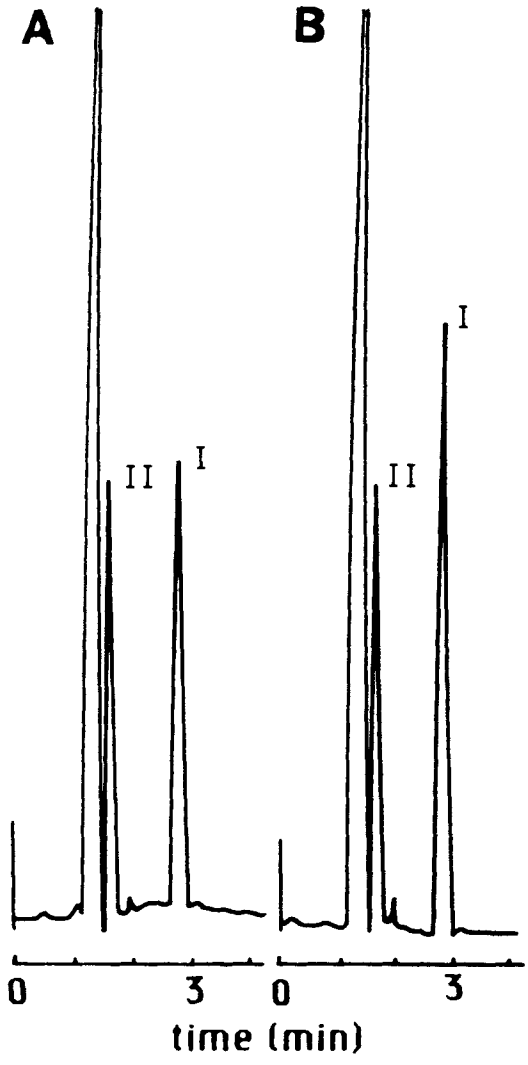

FIGURE 1.

Chromatograms of AT (I) in rat liver microsomes using ATA (II) as an internal standard. (A) Extract of blank microsomes; (B) extract of the same microsomes spiked with extra AT (1 μg/ml). Chromatograms were recorded using a programmable fluorescence detector set at 0.1 A.U. between 0 and 2 min and at 0.05 AU between 2 and 4 min. Auto-zeros were given at t=0 and t=2 min.

TABLE 1

Reproducibility of the Determination of alpha-Tocopherol in Microsomes or Buffer Spiked at Different Concentrations (Mean ± S.D.).

Added concentration (μg/ml)	n	measured concentration (μg/ml)	coefficient of variation %	accuracy %	recovery %
0.100	3	0.092±0.001	1.1	92±1	100±1
0.250	3	0.245±0.002	0.8	98±1	103±1
0.500	3	0.511±0.006	1.2	102±1	106±4
1.000	3	0.978±0.012	1.2	98±1	100±3
1.500	3	1.507±0.040	2.7	100±3	103±2
2.000	3	2.000±0.098	4.9	100±5	101±5

TABLE 2

Levels of alpha-Tocopherol in Microsomes of Rat Liver and Brain (Mean ± S.D.).

	n	AT concentration (μg/ml)	AT concentration (μg/mg protein)
liver	9	1.14 ± 0.63	0.10 ± 0.06
brain	9	0.15 ± 0.06	0.12 ± 0.07

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