Journal of Chromatography, 495 (1989) 269–274
Biomedical Applications
Elsevier Science Publishers B V Amsterdam — Printed in The Netherlands

CHROMBIO 4923

Note

Improved high-performance liquid chromatographic technique for the determination of hepatic α-tocopherol

H R PATEL*, S P ASHMORE, L BARROW and M S TANNER

Department of Child Health, Clinical Sciences Building, University of Leicester P O Box 65, Leicester LE2 7LX (U K)

(First received April 7th, 1989, revised manuscript received June 13th 1989)

High-performance liquid chromatography (HPLC) has been used extensively for the determination of serum α-tocopherol in clinical and analytical laboratories, vitamin E deficiency having important implications for cell membrane peroxidative damage in preterm infants, neurological disorders and chronic liver disease It has recently been recognised, however, that low serum levels of α-tocopherol may not accurately reflect tissue concentrations, particularly in the presence of liver disease [1] HPLC assays for analysis of tissue α-tocopherol have been beset by problems of incomplete extraction in hexane [2], and whilst a more complete extraction is achieved with acetone, a large solvent front containing interfering substances may make it difficult to measure accurately tissue α-tocopherol We present a method for quantitative determination of α-tocopherol extracted with acetone from small samples of rat liver, which utilises a simple Sep-Pak C_{18} cartridge purification and elution with ethanol, prior to separation of the rat liver homogenates on a C_8 Ultrasphere column by HPLC

EXPERIMENTAL

Animals

Inbred male rats (Wags, Harlan Olac, Oxon, U K) weighing approximately 125 g, were fed either a vitamin E-deficient diet (0 5 mg/kg) (SDS, Essex,

0378-4347/89/$03 50

U K) or the same diet supplemented with vitamin E (100 mg/kg) to normal levels After four weeks the rats underwent a lobectomy to provide 200 mg wet weight liver for duplicate vitamin E analysis The liver was frozen at −20°C for upto seven days

Reagents

d-α-Tocopherol and *d*-α-tocopherol acetate were obtained from Sigma (Poole, U K) Methanol (HPLC grade), ethanol and acetone (AR grade) were from Fisons (Loughborough, U K)

Apparatus and conditions

The HPLC equipment consisted of a Model LC3-XP pump (Pye Unicam, Cambridge, U K) and a Magnus M7110 pneumatic autosampler equipped with a 200-μl loop Chromatography was performed on an Ultrasphere C_8 column (250 mm × 4 6 mm I D , 5 μm, Beckman, Bucks, U K) using pure methanol as

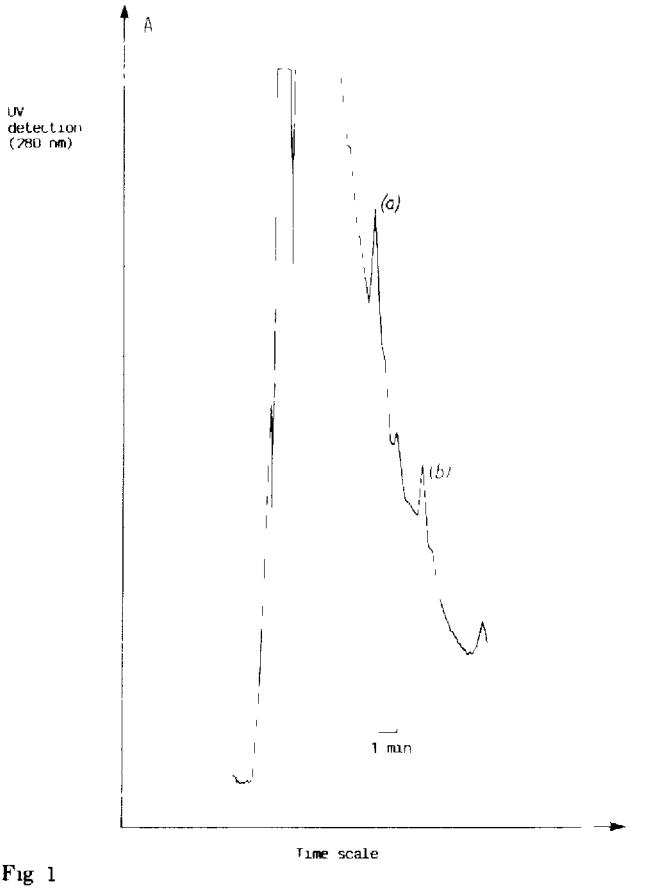

Fig 1

Fig 1 High-performance liquid chromatograms of the separation of α-tocopherol (a) and α-tocopherol acetate (b) from rat liver (A) without prior treatment and (B) following a Sep-Pak C_{18} cartridge separation

the mobile phase, at a flow rate of 1 5 ml/min The absorption was monitored at 280 nm using a UV detector (Pye Unicam, Cambridge, U K) and peak heights were recorded on a 4270 integrator (Spectra-Physics, Hemel Hempstead, U K)

Standards

A stock solution of *d*-α-tocopherol (10 mg/ml) and a stock solution of internal standard, *d*-α-tocopherol acetate (10 mg/ml) were prepared in ethanol on the day of analysis of hepatic α-tocopherol and diluted in ethanol to give five standards with a range of 2 5–50 0 μg/ml *d*-α-Tocopherol acetate was added to each standard to give a concentration of 5 μg/ml These standards

were taken through the assay, as described below for liver preparation, resulting in standards with a final concentration ranging between 0 25 and 5 00 μg/ml

Sample preparation and chromatography

Samples of rat liver (100 mg) were homogenised on ice for 1 min in 2 ml acetone The homogenate was centrifuged for 10 min at 1200 *g* and the supernatant decanted A further two acetone extractions were performed and the three supernatant extracts pooled prior to evaporation under nitrogen at 60°C The dry residue was redissolved in 1 ml of methanol

A Sep-Pak C_{18} cartridge (Millipore, Middlesex, U K) was prepared by passage of 4 ml of methanol followed by 4 ml of distilled water, using a siliconised glass syringe The standards and reconstituted samples were slowly passed into the cartridge and the cartridge was washed through with 2 ml of distilled water The tocopherol isomers, the internal standard and molecules of similar polarity were subsequently collected from the cartridge by passing through 2 ml of ethanol The ethanol eluent was then evaporated to dryness under nitrogen, reconstituted with 1 ml of methanol and 200-μl aliquots were injected onto the HPLC column

RESULTS

Calibration curves

Calibration curves ($n=6$) obtained from the peak height ratio of *d*-α-tocopherol/*d*-α-tocopherol acetate in a range of standard concentrations gave a linear regression equation of $y=0\ 0289+0\ 0560x$ and a coefficient of correlation of $r=0\ 996$

Sep-Pak purification

The elution profile of liver homogenate containing *d*-α-tocopherol acetate internal standard is shown in Fig 1 Insufficient resolution was obtained by direct HPLC analysis of liver from vitamin E-replete rats (Fig 1A) A prior Sep-Pak C_{18} cartridge separation step, however, enabled the removal of molecules with a relative polarity both greater and lesser than α-tocopherol (Fig 1B)

Tocopherol isomer identification

Vitamin E is comprised of four compounds α-, β-, γ- and δ-tocopherol In order to confirm, therefore, that α-tocopherol is the isomer detected by this assay, each isomer was separately passed through the HPLC system and the retention time recorded, and an elution profile of a mixture of these isomers subsequently obtained showing resolution of the tocopherol compounds (Fig 2)

Fig 2 High-performance liquid chromatogram of standard tocopherol isomers at a concentration of 2 μg/ml Peaks 1 = δ-tocopherol, 2 = γ-tocopherol and β-tocopherol co-eluting, 3 = α-tocopherol

Recovery

The recovery of α-tocopherol was estimated by supplementing the liver with a known amount of *d*-α-tocopherol standard before homogenisation, and the α-tocopherol concentration was measured compared to the spike added The average recovery at 10 μg/ml was 95 4 $\pm$ 1 7% (n = 10)

Detection limit

The detection limit was 0 5 μg α-tocopherol per gram liver

Precision

The within-day and day-to-day coefficients of variation were 2 3% (n = 8) and 2 8% (n = 12), respectively

Normal values

The retention times for *d*-α-tocopherol and *d*-α-tocopherol acetate were approximately 7 and 10 min, respectively High-molecular-mass molecules eluted

upto approximately 40 min, necessitating two methanol washes of the column between each liver sample. Normal values (mean ± S.D.) obtained in vitamin E-replete rats were 28.9 ± 3.7 μg α-tocopherol per gram liver, whilst values were reduced to 3.7 ± 1.5 μg/g in rats fed a vitamin E-deficient diet for four weeks.

DISCUSSION

The method presented for analysis of hepatic α-tocopherol is simple, precise and sensitive to 0.05 μg α-tocopherol per gram tissue. It enables analysis of as little as 0.1 g wet weight of liver, even when the rats are almost completely vitamin E-deficient. Extraction with acetone enables a good recovery of tissue α-tocopherol and use of a Sep-Pak C_{18} cartridge, to remove molecules with a polarity greater than α-tocopherol and retain relatively non-polar molecules, enables a much improved resolution of the HPLC peaks.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr. D.B. Morton and Jenny Rees for performing the rat lobectomies and to staff of the animal house for care and maintenance of the rats. L.B. was supported by the International Copper Research Association.

REFERENCES

1 L. Barrow, H.R. Patel, S.P. Ashmore and M.S. Tanner, J. Pediatr. Gastroenterol. Nutr., 9 (1989) in press.
2 B.J. Zaspel and A.S. Csallany, Anal. Biochem., 130 (1983) 146.