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Short communication

# Determination of vitamin E in human plasma by high-performance liquid chromatography

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**Abstract**

The use of selective protein precipitation to enhance the recovery of vitamin E from plasma, by minimising binding with very-low-density lipoproteins, is reported. The procedure employed treatment of plasma with magnesium chloride and tungstate, followed by methanol protein precipitation. Separation of vitamin E was performed using reversed-phase high-performance liquid chromatography of the methanol extracts with subsequent UV detection of the compound. Using this technique the procedure was observed to be specific for vitamin E and linear over the range 1.0 to 40.0 μg/ml. The within-run imprecision (C.V.) at three different supplemented plasma vitamin E concentrations of 5.0, 10.0 and 20.0 μg/ml was 4.51, 3.33 and 2.58%, respectively, and the between-run imprecision (C.V.) estimated to be 5.19, 3.69 and 3.67%, respectively. With the same supplemented plasma vitamin E concentrations, the overall accuracy (bias) of the procedure, using an albumin matrix for calibration, was estimated to be 6.0, −5.0 and −3.5%, respectively, and the recovery of vitamin E from six different spiked plasma samples estimated to be 98.2±2.6%.

*Keywords:* Vitamins

## 1. Introduction

Fat soluble vitamin E [(±)-α-tocopherol] is of dietary importance in man and is transported in plasma mainly associated with chylomicrons and very-low-density lipoproteins (VLDL), and rapid exchange of the compound occurs between red cell membranes and plasma.

Common methods for the estimation of the fat soluble vitamins have usually employed protein precipitation with ethanol, saponification by alkaline hydrolysis and highly non-polar organic solvent extraction prior to high-performance liquid chromatography (HPLC) analysis [1,2]. Incubation with lipase prior to solvent extraction has also been utilised in food products [3]. In either case, heat or alkaline conditions are employed in an effort to remove or minimise matrix effects due to binding of vitamin E to lipoproteins. This is not conducive to accurate measurement of the compound since, in the presence of oxygen, vitamin E is unstable to heat and alkaline conditions. Regardless of these extreme conditions, vitamin E is also slowly oxidised and is light sensitive.

This paper reports the development of a simplified sample preparation procedure prior to HPLC analysis for the determination of vitamin E in human plasma. The sample preparation procedure utilises the prop-

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erties of divalent and polyvalent ions to selectively precipitate VLDL from high density lipoproteins [4,5] and as a consequence the potential release of vitamin E from lipoproteins. In this manner, pretreatment of plasma with magnesium chloride and sodium tungstate, prior to protein precipitation with methanol, minimises matrix effects and enhances recovery of vitamin E from plasma. In order to negate calibration difficulties due to the physiological presence of vitamin E in plasma, the use of a purified albumin solution as a substitute matrix is investigated.

## 2. Experimental

### 2.1. Instrumentation

Unless otherwise stated all of the HPLC and autosampling units were obtained from Anachem (Luton, UK). An isocratic HPLC system was used comprising a Gilson 306/5SC pump, a 118 UV detector and a Rheodyne 7010 injection valve fitted with a 100-μl loop on a 231 autosampler unit. Control of the HPLC system, integration of chromatographic peaks and communication with the 231 unit (via Gilson Medical Electronics GSIOC) was made using a 715, V1.2, system controller (IBM PS1 with hard disc, EGA graphic card, mouse, MS DOS and Windows software V3.11).

### 2.2. Reagents

#### 2.2.1. General reagents

All chemicals were analytical grade obtained from Sigma Chemical Company (Poole, UK). HPLC grade water, prepared using a Purite (Thame, UK) system, was used for all reagent preparations. HPLC solvents were obtained from Romil (Cambridge, UK). The following stock reagents were prepared: (1) sodium tungstate–magnesium chloride solution (0.06:1.0 mol/l); (2) 80% (v/v) methanol in water.

#### 2.2.2. Standard preparations

A 2000 μg/ml solution of vitamin E in methanol was prepared which appeared stable for two weeks in the dark at 4°C. Working standards (50, 100, 250, 500 and 1000 μg/ml) were prepared by diluting the stock solution in methanol. Calibration standards (ranging from 1.0 to 40.0 μg/ml) were prepared fresh by supplementing bovine albumin solution (50 g/l) with the working standards.

### 2.3. Chromatographic conditions

An isocratic elution was used comprising methanol–acetonitrile–water (50:35:15, v/v) at a flow-rate of 1.5 ml/min. The HPLC column (100×4.6 mm I.D.) was packed with 5 μm particles of Kromasil $C_1$ (Technicol, Stockport, UK). The analytical column was maintained at ambient temperature. The detector was set at 292 nm wavelength and absorbance range of 0.002 AUFS.

### 2.4. Sample preparation

Sample preparation and HPLC injection was optimised as follows:

1. 100 μl of plasma or calibrant was vortex-mixed with 20 μl of tungstate–magnesium chloride solution.
2. 1.0 ml of methanol was added and after vortex-mixing the precipitated proteins were removed by centrifugation.
3. 200 μl of supernatant was aspirated into an autosampler vial.
4. 100 μl of the supernatant was automatically injected onto the HPLC column and the autosampler probe and lines purged with 2.0 ml of 80% methanol.

### 2.5. Quantification

Calibration standards were situated at the beginning and end of each analytical run. A linear regression (weighting $1/X^2$) was performed on the peak areas and concentrations of both sets of standards. The regression line established was used to calculate test analyte concentrations.

### 2.6. Quality control

A pooled heparinised plasma sample (obtained from healthy volunteers) was supplemented with 5.0,

10.0 and 20.0 μg/ml of vitamin E prepared in methanol. These quality control (QC) samples were aliquoted and stored at −20°C, together with the basal plasma used for supplementing. The vitamin E solutions used to supplement the plasma were prepared from separate solutions to those used for preparation of the calibration standards. A further quality control sample was prepared containing 1.0 μg/ml of vitamin E in bovine albumin solution.

## 3. Results and discussion

### 3.1. Chromatography conditions

Fig. 1a shows a chromatogram obtained using this method to analyse blank albumin solution. Fig. 1b and Fig. 1c show chromatograms obtained after analysing the basal plasma sample and the same plasma sample supplemented with 10.0 μg/ml of vitamin E respectively. A short-chain $C_1$ column was utilised to reduce retention times and improve sample throughput.

### 3.2. Optimisation of calibration and plasma treatment

Difficulties arise with calibration procedures where the compound is present in the matrix under investigation, and as a consequence it is useful to identify suitable blank matrix substitutes that behave, preparatively, in a similar manner to that being analysed.

Using a non-protein matrix the slope of the vitamin E regression line parameters differed from protein matrices by a factor of 0.8 and these matrix differences indicate that calibration standards must be prepared in a protein base for accurate vitamin E estimations in plasma if a substitute matrix is to be employed. These differences may be due to volume changes when adding methanol to aqueous media (i.e. non-protein) during sample preparation. The addition of a suitable internal standard may circumvent this observational difference, but in its absence a protein base is required as a substitute for plasma if the desired accuracy is to be achieved.

Further difficulties must be overcome if the effect of protein binding of vitamin E is to be negated and

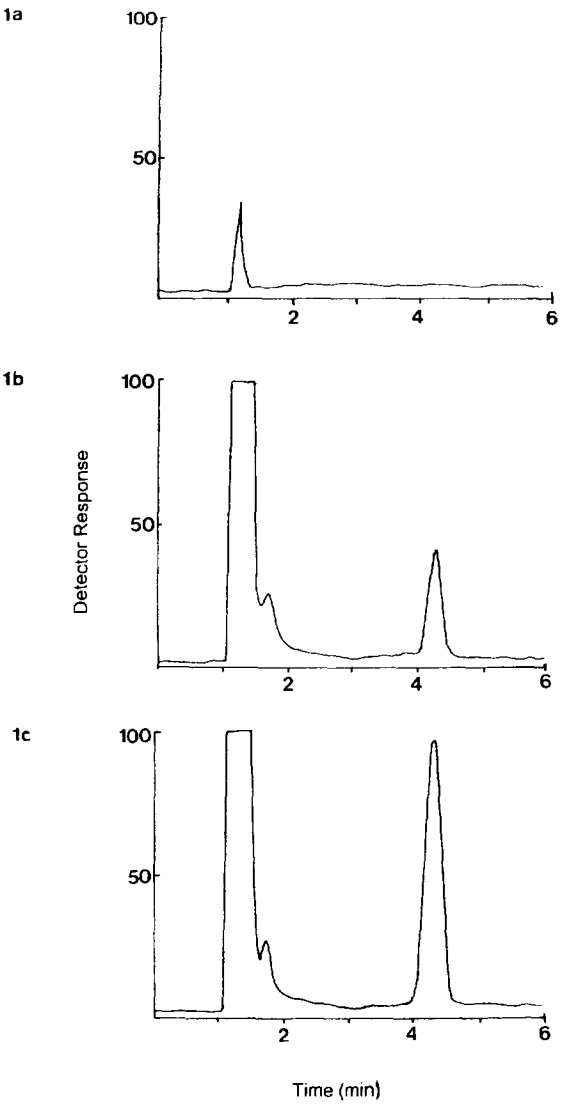


Fig. 1. (a) Chromatogram of blank albumin solution. (b) Chromatogram of basal plasma with a measured vitamin E concentration of 12.5 μg/ml. (c) Chromatogram of basal plasma supplemented with 10.0 μg/ml of vitamin E.

the recovery of vitamin E from albumin solution is to be identical to that from plasma containing VLDL. During investigations into this problem, the use of divalent ions proved to offer a simple and viable procedure to release vitamin E from VLDL. Moreover, without the addition of magnesium chloride–tungstate solution to plasma, prior to protein precipitation, a bias of −60.0% was observed when re-

Table 1
Within and between-run imprecision and overall accuracy of the vitamin E method

| Nominal vitamin E concentration (μg/ml) | Measured vitamin E concentration (μg/ml) | Coefficient of variation (%) | | Accuracy (% bias) |
|---|---|---|---|---|
| | | Within-run | Between-run | |
| 0 (basal) | 12.8 | – | – | – |
| 1 | 1.03 | 5.59 | 7.68 | -[a] |
| 5 | 18.1 | 4.51 | 5.19 | 6.0 |
| 10 | 22.3 | 3.33 | 3.69 | −5.0 |
| 20 | 32.1 | 2.58 | 3.67 | −3.5 |

[a] Refer to Section 3.3.2.

covery of vitamin E in plasma was compared with its recovery from albumin.

### 3.3. Assay performance

#### 3.3.1. Linearity and analytical range

Using calibration albumin solutions supplemented with vitamin E, peak areas varied linearly over the analytical range employed and the lower limit of quantification was set at the lowest standard concentration on the calibration curve.

#### 3.3.2. Imprecision

The within-run coefficient of variation (C.V.) was estimated by assaying the quality control samples ten times in the same analytical run. The between-run (C.V.) was obtained by estimating six replicates each of the same control samples in a further three analytical runs. The results are shown in Table 1 and demonstrate an acceptable assay performance, but because of the physiological presence of vitamin E in plasma, the imprecision at the lower limit of quantification was determined by estimating replicates of albumin solution supplemented with 1.0 μg/ml vitamin E. As a consequence, accuracy of the assay was not calculated at this concentration.

#### 3.3.3. Accuracy

The overall per centage bias of the assay was obtained by measuring six replicates of the basal plasma vitamin E concentrations in the same three analytical runs, used to evaluate the between-run imprecision, and subtracting the measured mean concentration from the measured mean plasma vitamin E concentration in the QC samples (Table 1). This data indicates that the inclusion of divalent ions minimised plasma VLDL binding sufficiently to allow the use of an albumin matrix for calibration purposes.

The analytical recovery of the method was also examined by supplementing six different plasma samples with a nominal vitamin E concentration of 10.0 μg/ml and analysing both the supplemented and basal plasma samples using the method described. After subtracting the basal vitamin E from the total estimated concentration, the analytical recovery was found to be 98.2±2.6%.

### 3.4. Reference range

The basal plasma vitamin E concentration ranged from 9.5 to 18.1 μg/ml. This data was obtained from measuring six human plasma samples from healthy volunteers. This is in general agreement with a quoted reference interval [4].

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