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APPLICATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC FLUORESCENCE METHOD FOR THE RAPID DETERMINATION OF α-TOCOPHEROL IN THE PLASMA OF CATTLE AND PIGS AND ITS COMPARISON WITH DIRECT FLUORESCENCE AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–ULTRAVIOLET DETECTION METHODS

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SUMMARY

High-performance liquid chromatography is used to develop a sensitive method for the determination of tocopherol levels in the plasma of cattle and pigs. This method is compared with a similar method using UV detection and one using direct fluorescence determination of tocopherol.

Finally a double injection technique used in conjuction with fluorescence detection is shown to enhance the rate of analysis of the tocopherol levels in bovine plasma extracts.

INTRODUCTION

Vitamin E and particularly α-tocopherol offers protection against a number of animal diseases, such as mulberry heart, hepatosis dietetica and muscular dystrophy in pigs[1,2] and nutritional myopathy in young cattle[3,4]. Selenium also offers protection in these diseases probably through its involvement in the function of the seleno-enzyme glutathione peroxidase (GPX) (EC 1.11.1.9).

At present the selenium status of cattle and pigs may be readily assessed by analysis of the red cell for its GPX activity. There is at present no rapid means of assessing the vitamin E status of these animals, which is necessary in order to determine the interactions between the two protectants in the disease state.

The determination of plasma or serum α-tocopherol levels in serum or plasma offer a means for determining the vitamin E status of the animal. This seems to have some potential as the tocopherol level has been shown to be depleted in clinically affected cattle[3,4]. Because of the length of time involved in carrying out analysis by traditional colorimetric or gas chromatographic methods it has been impractical to monitor the status of animals for the vitamin.

High-performance liquid chromatography (HPLC) offers the possibility of combining rapid analysis with the separation of tocopherols from interfering substances. This technique with UV detection has been used for the determination of tocopherols in human serum[5,6]. The level of α-tocopherol in human serum, however,

is an order of magnitude greater than the levels in clinically normal pigs and cattle and can be nearly two orders of magnitude higher than those in clinically affected animals[4]. The requirements for sensitivity and freedom from interference from other substances are therefore more critical for the determination of tocopherol in animal sera or plasma. The tocopherols are natural fluorphores and therefore a combination of HPLC with fluorescence detection offers the possibility of developing a rapid analytical procedure of the required sensitivity and specificity for the determination of tocopherol levels in the serum of domestic anaimals.

The developed method is compared with the same HPLC technique using absorbance detection, along with a direct fluorescence technique[7].

EXPERIMENTAL

Apparatus

The high-performance liquid chromatograph pump (Model 6000), injector (Model U6K), UV absorbance detector (Model 440), column (μBondapak C_{18}, 300 × 3.9 mm) and pre-column filled with C_{18}/Corasil (used to protect the main analytical column) were purchased from Waters Assoc. (Hartford, Great Britain). The fluorescence spectrophotometer (Model 204) and flow cell (100 μl) were purchased from Perkin-Elmer (Beaconsfield, Great Britain). The chart recorders used were either a single-channel potentiometric recorder purchased from J. J. Lloyd Instruments (Southampton, Great Britain) or a dual-channel R520 Servoscribe from Smiths Instruments (Cricklewood, Great Britain).

The fluorimeter was connected in series with the UV absorbance detector so that a dual trace of absorbance and fluorescence could be obtained where required.

Samples containing organic solvents were concentrated over nitrogen on a thermostated dri-block (Dri-Block Heater, Gallenkamp, Widnes, Great Britain).

All injections to the HPLC were carried out with appropriate Hamilton syringes which were obtained from Waters Assoc.

Reagents

AnalaR grade methanol and ethanol were obtained from Hopkin and Williams (Ramford, Great Britain). *n*-Hexane was obtained from BDH (Poole, Great Britain). The *n*-hexane and methanol were re-distilled before use to remove fluorescent impurities. *d*-α-Tocopherol was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.) and was used without further purification.

Heparinized blood samples were obtained from the jugular vein from both cattle and pigs under a variety of dietary conditions in order that a range of α-tocopherol levels in blood might be examined.

Procedure

Serum (1 ml) was pipetted into a 100 × 16 mm glass stoppered tube. If recovery experiments were being carried out α-tocopherol was added at this stage. Ethanol (1 ml) was added with mixing to precipitate proteins followed by 5 ml of hexane. The tube was shaken vigorously for 1 min and the layers were allowed to separate. If necessary a low speed centrifugation (1000 *g*) was used to clarify the solvent layer. Four millilitres of the hexane layer were pipetted off for analysis.

For direct fluorescence determination of topocherol the hexane layer was transferred to the fluorimeter and measured with an excitation wavelength of 296 nm and an emission wavelength of 330 nm. The hexane was drawn continuously through the flow cell using a simple peristaltic pump to avoid photodecomposition of the tocopherol.

Ultraviolet absorption measurements were made at 280 nm.

In the HPLC methods the hexane extract was evaporated to dryness on the Dri-block at 70° under a nitrogen atmosphere. The residue was redissolved in 100 μl of *n*-hexane and an appropriate aliquot applied to the column (25 to 50 μl). Redissolved extracts were maintained at 4° in an ice-bath prior to analysis to limit evaporative volume changes.

The mobile phase used on the HPLC column consisted of methanol–water (97:3). The flow-rate was 3 ml/min. The column was calibrated by injecting 25 μl of a 20 μg/ml solution of α-tocopherol (0.5 μg) in *n*-hexane.

The concentration of α-tocopherol was related linearly to the height of either the absorbance or fluorescence peak.

RESULTS

A typical chromatogram using the HPLC–fluorescence technique is illustrated in Fig. 1a for three samples: I_1, tocopherol standard; I_2, bovine plasma extract; and I_3, pig plasma extract. Using fluorescence detection tocopherol has an apparent retention time of 4 min.

The bovine and pig plasma extracts show various fluorescent compounds eluting prior to tocopherol, but none eluted subsquent to tocopherol. Pig plasma extracts showed a consistently greater number of compounds eluting from the column compared with bovine samples.

In contrast to the limited number of peaks detected using fluorescence detection, considerably more peaks appear using UV absorption detection and their absorbance relative to the absorbance of tocopherol is much more intense.

Fig. 1b illustrates the absorbance profile obtained with the same samples used to obtain the fluorescence elution pattern obtained in Fig. 1a. The profile is rather complex as the elution pattern for the pig plasma extract I_{11} is interspersed between the initial elution pattern in which tocopherol elutes, for the bovine sample I_{10}, and the elution of the carotene, present in the bovine sample. With UV detection the tocopherol elutes with an apparent retention time of 4 min while carotene has an apparent retention time of 19 min.

In the pig samples γ-tocopherol is sometimes present and elutes immediately before α-tocopherol.

The within day precision of the HPLC fluorescence method (Table I) was determined using two pooled serum samples having differing concentrations of tocopherol.

Extraction efficiencies were routinely determined by doping serum samples with α-tocopherol dissolved in ethanol. The results of a series of extraction experiments carried out over a period of time for both pig and bovine sera are summarized in Table II.

Three different methods of determining α-tocopherol were compared in two

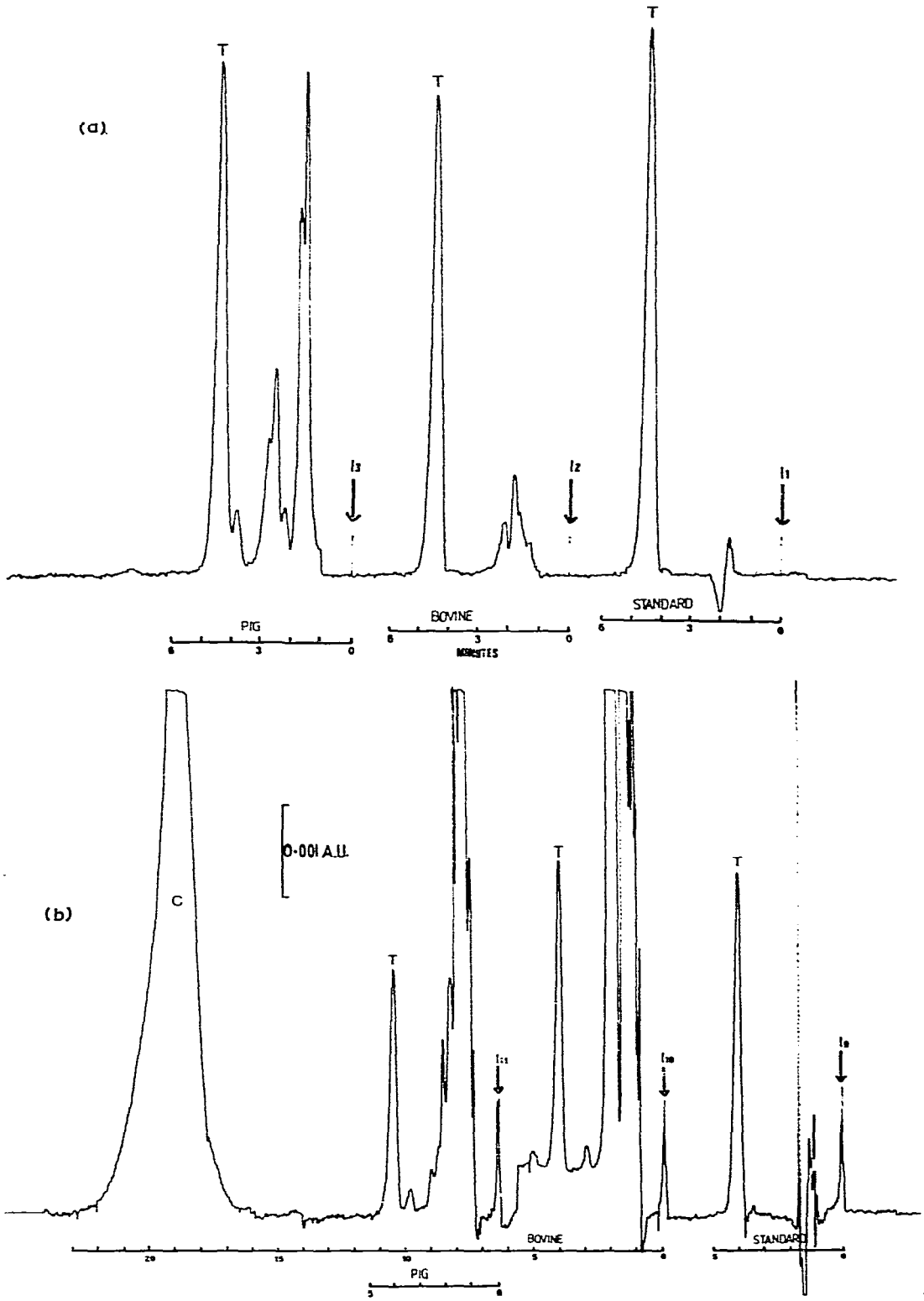

Fig. 1. Fluorescence (a) and ultraviolet (b) detection of α-tocopherol. Three samples were used 0.5 μg of α-tocopherol standard (I_1 and I_9) and extracts of bovine (I_2 and I_{10}) and pig (I_3 and I_{11} plasma. The tocopherol (T) and the carotene (C) peaks from the bovine sample are identified as i the point of injection.

TABLE I

ANALYTICAL PRECISION OF THE HPLC–FLUORESCENCE PROCEDURE FOR α-TOCOPHEROL

Samples 1 and 2 were pooled bovine plasma. $n = 12$.

Sample	*Mean (μg/ml)*	*S.D. (μg/ml)*	*C.V. (%)*
1	0.96	0.049	5.1
2	2.20	0.113	5.1

TABLE II

RECOVERY OF ADDED TOCOPHEROL TO PIG AND BOVINE SERUM

2 μg of tocopherol in ethanol was added per ml of serum; detection: HPLC–fluorescence method. $n = 8$.

Serum	*Mean recovery (%)*	*C.V. (%)*
Bovine	97.3	7.7
Pig	93.6	7.3

TABLE III

TWO EXPERIMENTS TO COMPARE HPLC–FLUORESCENCE DETECTION WITH HPLC–UV AND DIRECT FLUORESCENCE METHODS FOR α-TOCOPHEROL

A set of 12 pig plasma samples were analysed by the three techniques in experiment 1 and by two techniques in experiment 2.

	Mean (μg/ml)	*S.D. (μg/ml)*	*Range (μg/ml)*	*Significance*
Experiment 1				
(1) HPLC–fluorescence	1.34	0.57	0.67–2.27	
(2) HPLC–UV	1.65	0.36	0.99–2.67	
(3) Direct fluorescence	2.73	0.59	1.97–3.61	
Difference 2 − 1	0.31	0.046		$P < 0.005$
Difference 3 − 1	1.39	0.118		$P < 0.005$
Difference 3 − 2	1.08	0.138		$P < 0.005$
Experiment 2				
(1) HPLC–fluorescence	0.86	0.40	0.33–1.74	
(2) Direct fluorescence	1.76	0.56	1.00–3.24	
Difference 2 − 1	0.90	0.310		$P < 0.005$

seperate experiments: (1) direct fluorescence, (2) HPLC with fluorescence detection, or (3) HPLC with UV detection. Table III summarises the difference between the methods. The results obtained were compared by means of the paired *t*-test in order to obtain the average discrepancy in apparent topopherol concentration between the methods. Examination of the results demonstrates that the direct fluorescence method gives a considerable higher value for tocopherol than either the HPLC fluorescence or the HPLC–UV methods. This is not surprising in light of the number of fluorescent compounds eluted from pig serum (Fig. 1). The HPLC–UV method also gives a higher tocopherol result than the HPLC fluorescent method. This experimental result confirmed our observations when running the output from the fluorescence and

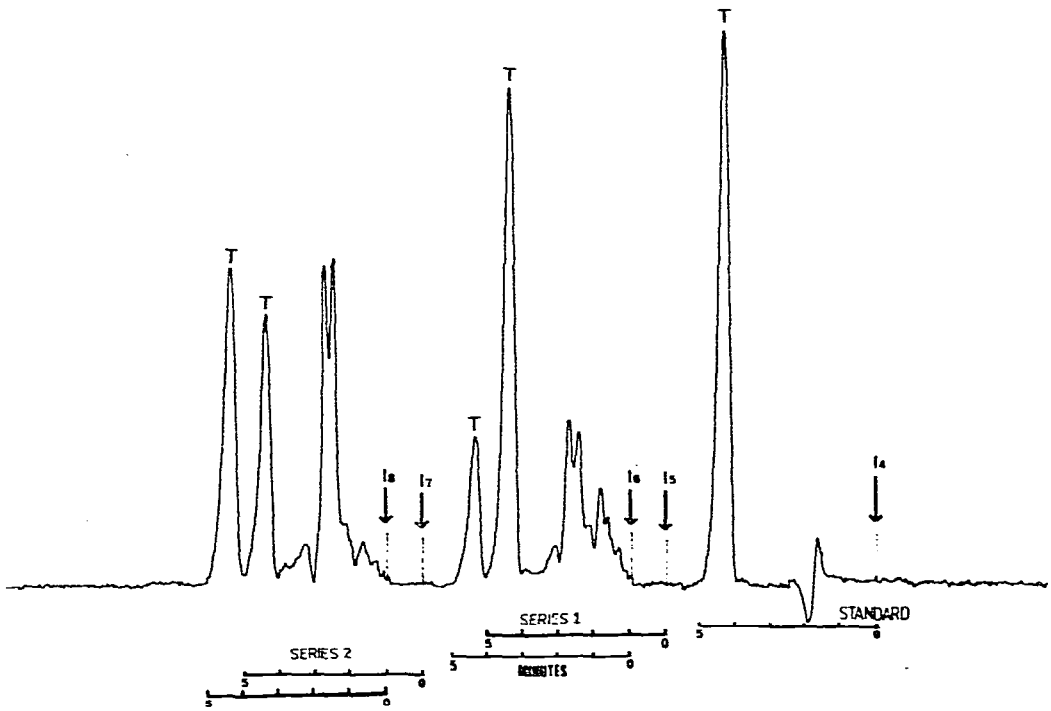

Fig. 2. A double injection fluorescence technique for the determination of tocopherol in bovine plasma extracts. The elution pattern consists of a tocopherol standard (0.5 μg) along with 4 plasma extracts (I_5–I_8). The tocopherol peaks (T) and the points of injection are identified.

absorbance detectors on the same recorder when it was observed that the ratio of absorbance to fluorescence peaks for tocopherol were different than that obtained for the standard.

The observation that only a few fluorescent compounds elute early from bovine plasma extracts has been used to devise a dual sample injection technique. Two samples are injected sequentially with an interval of 1 min, *i.e.* during the same chromatographic elution.

Fig. 2 illustrates the elution pattern obtained from using this technique for the determination of tocopherol levels in four samples (I_5–I_8).

The accuracy of this dual sample technique was compared with that of the single sample injection technique. Table IV summarises the results obtained in determining the tocopherol levels in a number of bovine plasma extracts.

TABLE IV

COMPARISON OF DOUBLE AND SINGLE SAMPLE INJECTION FOR THE DETERMINATION OF α-TOCOPHEROL IN BOVINE PLASMA EXTRACTS

$n = 14$.

Method	*Mean (μg/ml)*	*S.D. (μg/ml)*	*Range (μg/ml)*	*Significance*
Double	1.42	0.662	0.7–2.6	
Single	1.41	0.635	0.7–2.7	
Difference	0.01	0.13		NS

DISCUSSION

The use of fluorescence detection in association with HPLC offers a number of advantages for the determination of tocopherol levels in bovine and pig plasma extracts. Interfering substances in the plasma extracts from both these species are resolved from tocopherol by the use of HPLC. Fluorescence detection further enhances the specificity of the method and the sensitivity is well within the range necessary for the detection of levels normally present in the deficiency states encountered in these species.

Because of interfering substances the application of UV detection and also of direct fluorescence methods should be treated with caution as they have shown erronously high levels. This is particularly true when a distinction is being made between deficiency and sub-optimal states.

The use of the double injection technique for the determination of tocopherol in bovine plasma samples offers one particular advantage in that the rate of analysis is increased by approximately 50%, without any apparent reduction in sensitivity or reproducibility.

The developed techniques is currently being used with a recently developed method[8], for the determination of α-tocopherol in feedingstuffs, in order to examine the status of the animal and the interaction of its status with the nutritional supply of the vitamin. This is of particular importance in assessing the interactions responsible for the occurrence of clinical disease in field outbreaks.

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