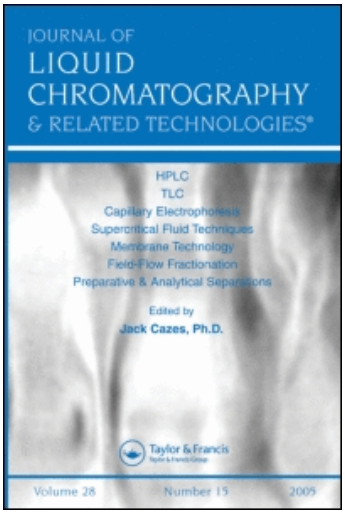

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A SELECTIVE LIQUID CHROMATOGRAPHY ASSAY FOR THE DETERMINATION OF d1-α-TOCOPHEROL ACETATE IN PLASMA SAMPLES

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method for the assay of tocopherol and tocopherol acetate in biological samples is described. Results was presented for the linearity, sensitivity and reproducibility. This HPLC method for tocopherol acetate was acceptable in terms of linearity, sensitivity and inter-day reproducibility and was convenient for the routine analysis of plasma samples.

INTRODUCTION

dl-α Tocopherol acetate (2,5,7,8, tetramethyl-2-(4',8',12',trimethyltridecyl)-6-chromanol acetate); **1** and dl-α-Tocopherol (2,5,7,8, tetramethyl-2-(4',8',12', trimethyltridecyl)-6-chromanol); **2**, figure 1, is

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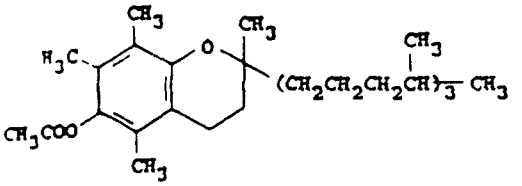

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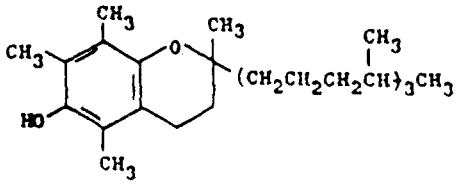

II

FIGURE 1. Chemical structures of Tocopherol acetate (I) and Tocopherol(II).

currently receiving attention concerning its efficacy in preventing or reducing the incidence of severe clinical conditions associated with adverse oxidation events in premature newborns (retolental fibroplasia, intraventricular haemorrhage, bronchopulmonary dysplasia, haemolytic anemia) and in adults (coronary, rheumatic and hypertensive heart diseases) [1,2]. When adminisnistred, the **1** is hydrolyzed to **2**, the active moiety. High plasma concentrations are obtained during three days following I.V. injections [3].In the USP XXII [4], a HPLC method have been developmen for tocopherol acetate determination

in pharmaceutical preparations. In recent years, numerous HPLC procedures have been developed for the determination of **2** in biological samples foods and pharmaceuticals [5-10]. Thus it is desirable to have an analytical method to cuantify the **1** and **2** in biological samples with both drugs, which could be readily applied to analyze several clinical samples daily.

EXPERIMENTAL

Material

dl-α-tocopherol and the dl-α tocopherol acetate were obtained from Merck (Darmstadt, Germany). HPLC grade methanol were purchased from Panreac (Spain). Distilled de-ionized water was used for the preparation of all aqueous solutions.

Instrumentation and Chromatographic Conditions

HPLC system was used, consisting of two Gilson (Middleton, WI, USA) 305 and 306 pumps, and a Gilson 231 XL automatic sampler attached to a injection valve (20μl sample loop), which was coupled to a 200 x 4.6 mm I.D. LiChrosorb RP-18 column, particle size 10 μm. The column was maintained at ambient temperature.

The mobile phase consisted of 100% methanol. The mobile phase was filtered through a Millipore HV LP

(Bedford, MA, USA) filter 0.45 μm prior to use. Total run time was 15 min. A flow-rate of 1.0 ml/min was employed. Injections were made 20 min apart to allow for complete column re-equilibration.

Detection of the analytes was accomplished using a Gilson 116 variable wavelength uv detector at 292 nm. Data were recorded on a Spectra-Physics SP4270 integrator (San Jose, CA, USA).

Animal

Male Beagle dogs (age 12 months; weight 10-12 Kg) were obtained from a recognized supplier, uniquely identified and housed individually in cages.

Sample Preparation

2 ml of methanol were added to aliquots (0.4 ml) of plasma to precipitate proteins. After vortex-mixing for 5 min, the plasma proteins were precipitated by centrifugation at 1200 g for 10 min and filtered through a 0.45 μm Millipore HV filter (Bedford, MA, USA). A 20 μl aliquot was injected onto the HPLC column.

Treatment of Analytical Data

The gradients, intercepts of the calibration curves, and linearity of each calibration graph were obtained by regression analysis. The different retentions time were

calculated. The resolution between two chromatographic peaks (R) was calculated from the equation [1] as follows [4]:

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2} \qquad (1)$$

Where t_1 and t_2 are the retention times and W_1 and W_2 the width of the peaks, measured by extrapolating the relatively straight sides to the baseline.

The interception values is calculated by the following equation:

$$a \pm t\ S_a \qquad (2)$$

Where, t is the value of t-Student for n-2 degrees of freedon and a probability of 0.05; and S_a is the variance of the interception values. If the zero value is between these limits, the proportionally condition is achieved.

The confidence intervals for the slope of the line of regression is calculated by the following ecuation:

$$b \pm t\ S_b \qquad (3)$$

Where, t is the value of t-Student for n-2 degrees of freedom and a probability of 0.05; and S_b the variance of the slope.

Lineality test. It was evaluated by the standard relative desviation for the slope according to the following ecuation:

$$S_{b\ rel\ (\%)} = \frac{S_b}{b}\ 100 \qquad (4)$$

Detection limits (D.L.) were statistically calculated from the following equation [11]:

$$D.L. = S_0^2 \frac{n-2}{n-1}^{1/2} \cdot \frac{t_p}{b} \qquad (5)$$

Where n is the number of values, t_p is the value of Student t at P= 0.05 level of significance and (n-2) degrees of freedom, b is the gradient and S_0^2 is the variance characterizing the dispersion of the points with respect to the line of regression.

The limit for experimental detection is the lower concentration that can be found.

The analytical recovery was calculated from 100 x amount found/ amount added at these concentrations.

Stability of **1** and **2** were measured in different samples after storage at -20 C and room temperature. Stored samples containing **1** and **2** were analyzed and compared to freshly prepared standards. Linear regression analysis of percent analyte found versus storage time was performed.

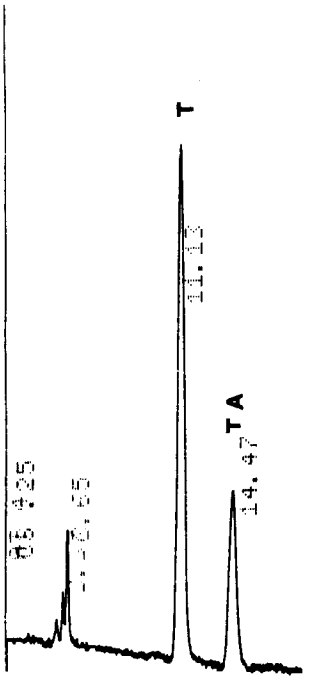

FIGURE 2. Chromatogram of Tocopherol acetate (**1**) and Tocopherol (**2**) in plasma samples.

RESULTS AND DISCUSSION

Chromatography

Figure 2 shows the HPLC chromatograms of **2** and **1** with retention times of 11.13 and 14.47 min respectively. The resolution between chromatographic peaks (R) for **1** and **2** was 1.33. This value allows good resolution without interference from **1** in analysis of **2**.

Linearity

The gradients, intercepts and linearity of each calibration graph were calculated and are summarized in table 1. The intercept values of **2** and **1** were not statistically ($P<0.05$) different from zero. Calibration curve for these methods were linear in the ranges tested, the linearity of **1** is higher than **2**.

The concentration range and detection limit are summarized in table 2. The detection limits evaluated by the statistical method were similar to those calculated according to the experimental method. The lowest detection limit calculated was obtained for **1** 0.17 $\mu g\ ml^{-1}$. The difference between the detection limits, "calculated" and "found" for **2** is probably due to the poorer linearity for this method.

Recovery

The absolute recoveries in plasma samples at these low, medium and high concentrations of **1** and **2** were evaluated. The analytical recovery were between 103.2-104.3% for **1** and between 96.7-102.6% for **2**. These results are provided in Table 3.

Specificity

Plasma was collected from 4 dog and screened for interference at the retention times of **1** and **2**. No

TABLE 1
Comparative Analytical Data for the Determination of dl-α-Tocopherol Acetate and dl-α-Tocopherol.

	dl-α-Tocopherol Acetate	dl-α-Tocopherol
Slopes $b \pm S_b$	1860.78±80.52	4879.82±440.09
Intercept $a \pm t\ S_a$	2829.10±54609.21	699.70±2696.78
Linearity $S_{b\ rel}$(%)	1.67	3.51

TABLE 2
Concentration Range and Detection Limit for dl-α-Tocopherol Acetate and dl-α-Tocopherol.

	dl-α-Tocopherol Acetate	dl-α-Tocopherol
Concentration Range ($\mu g\ ml^{-1}$)	0-50	0.10
Detection Limit Calculated ($\mu g\ ml^{-1}$)	0.17	0.59
Found ($\mu g\ ml^{-1}$)	0.20	0.75

TABLE 3
Recovery of dl-α-Tocopherol Acetate and dl-α-Tocopherol from plasma samples*.

Drug	Concentration (μg/ml)	% Recovery
dl-α-Tocopherol	1.0	104.3
	5.0	103.7
	10.0	103.2
dl-α-Tocopherol Acetate	12.5	96.7
	25.0	101.5
	50.0	102.6

(*) n=4

significant interference was observed with free plasma samples.

Inter and Intra-Day Precision

Due to the large concentration range of **1** required for this assay, standard curves were constructed in order to adequately quantitate unknown samples. Plasma concentrations (n=4) of 1.0, 5.0 and 10.0 μg/ml for **2**, the method yields relative standard deviations (RSD) of 4.68, 4.27 and 4.00% respectively; similar results were obtained by Miller and Vranderick [12] in inter-day precision of carbamazepine in human plasma. Plasma concentrations of 12.5, 25 and 50 μg/ml for **1**, the method yields RSD of 1.17, 1.21 and 0.61% respectively. Under the experimental conditions described the **1** was better than **2** method in the inter-day precision.

The intra-day precision was determined by the evaluation of a typical production run consistiny at concentrations of 12.5, 25.0 and 50.0 μg/ml of **1** and concentrations of 1.0, 5.0 and 10.0 μg/ml of **2**. The RSD of these samples were within 6.23%. These result are provided in table 4.

Application to Pharmacokinetic Study

The method was successfully been applied to a pilot pharmacokinetic study in dog. Figure 3, represents the

TABLE 4

(a) Inter-Day Precision in Plasma.

Drug	Concentration (μg/ml)	n	% RSD
dl-α-Tocopherol	1.0	4	4.68
	5.0	4	4.27
	10.0	4	4.00
dl-α-Tocopherol Acetate	12.5	4	1.17
	25.0	4	1.21
	50.0	4	0.61

(b) Intra-Day Precision in Plasma.

Drug	Concentration (μg/ml)	n	% RSD
dl-α-Tocopherol	1.0	4	6.32
	5.0	4	5.78
	10.0	4	4.87
dl-α-Tocopherol Acetate	12.5	4	2.27
	25.0	4	2.07
	50.0	4	1.97

plasma **1** and **2** concentration versus time profile, by a I.V. injection at 8 mg/kg of a **1** solution to a male beagle dog. All samples were analyzed by the method presented here.

Stability

1 and **2** appear to be stable in plasma upon storage at -20 C for up to 1 year, similar findings have been reported by Constock et al [13]. Plasma samples of **2** and **1** presented retention time of 10.24 and 13.62

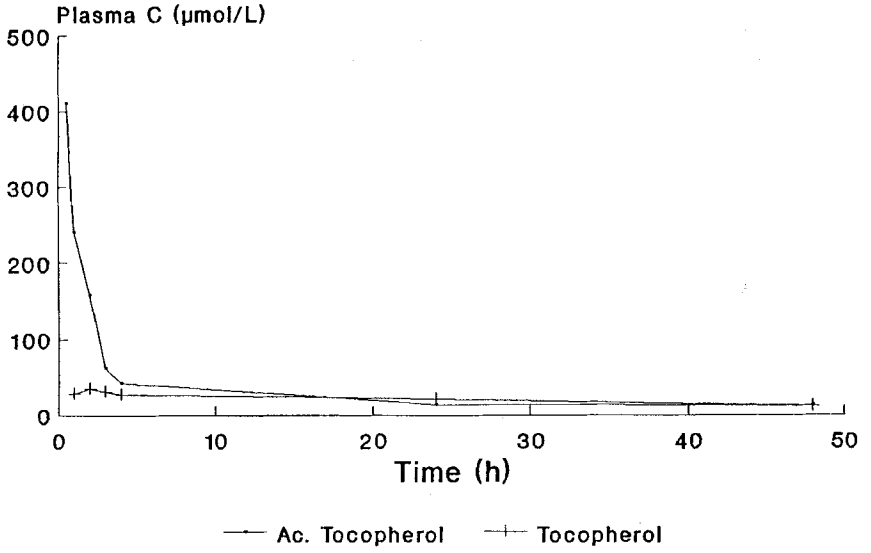

FIGURE 3. Representative plasma concentration-time profile of Tocopherol acetate and Tocopherol after a single 8 mg/Kg i.v. of Tocopherol acetate solution.

respectively, similar to the figure 2 and their plasma concentrations for **1** and **2** were inside of the representation concentration versus time profile (figure 3). Examination of **1** and **2** stability in mobile phase following extraction indicated to be stable upon storage at 4 C for up to 48 h, with RSD values of 1.6% for **2** and 2.8% for **1**. Therefore, to ensure sample integrity; HPLC analysis should be finalized before 48h.

Conclusions

Compared to other chromatographic procedure, this HPLC method has the advantage to be a very simple and

rapid procedure. Complete pharmacokinetic characterization of these drugs requires a sensitive and selective assay for both drug in plasma samples. We report here such an assay for the analysis of **1** and **2** from dog plasma. The major advantage of this method is the direct analysis without the need for a previous treatment of the different plasma samples.

Under the experimental conditions described the linearity, detection limit and recovery was best in tocopherol acetate that tocopherol free analyses. Inter and intra-day precision, expressed as the relative standard deviation (RSD), was always lower than 6.32%.

For the selectivity of the procedure and his rapidity, this HPLC assay is quite suitable for routine analysis in pharmacokinetic study.

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