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Spectrophotometric determination of vitamin E (α-tocopherol) using copper(II)–neocuproine reagent

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Abstract

The possibility of the utilization of the copper(II)–neocuproine spectrophotometric method, which has previously been shown to permit the determination of various reducing agents, to the determination of vitamin E was investigated. The molar absorptivity for vitamin E was found to be $(2.1 \pm 0.1) \times 10^4$ l mol^{-1} cm^{-1} and Beer's law was obeyed between 2.4×10^{-6} and 9.0×10^{-5} M concentrations of α-tocopherol. The relative standard deviation of the slope of the absorbance vs. concentration plot was 2.1%. The results obtained by the copper(II)–neocuproine method were compared with those achieved by both the standard HPLC and the widely used iron(III)–bathophenanthroline method by means of a *t*-test which showed that the precision of the developed method was not essentially different from those of the others. The developed method was successfully applied to three commercial samples, two in dragée and one in ampoule form. The α-tocopheryl acetate contained in the samples, which did not respond directly to the Cu(II)–neocuproine reagent, was subjected to alkaline hydrolysis prior to the analysis of the hydrolysis product, i.e., α-tocopherol. The molar absorptivity due to Cu(I)–neocuproine at 450 nm against a reagent blank indicated a two-electron oxidation of vitamin E by Cu(II)–neocuproine, which may be slightly enhanced by solvent effects. Copper(II)–neocuproine is an oxidant of strength comparable to that of Fe(III)–bathophenanthroline. The developed method, although less sensitive, is easy to use in conventional laboratories, unlike the Fe(III)–bathophenanthroline method, which requires specially prepared reagents and solvents. The method is free from interferences from such common reductants as ascorbic acid and Fe(II) salts, found in pharmaceutical formulations, after washing the formulation with water and collecting vitamin E in the ether extract for subsequent analysis.

Keywords: Copper(II)–neocuproine reagent; Spectrophotometry; α-Tocopherol; Vitamin E

1. Introduction

Vitamin E, also known as α-tocopherol, is an important vitamin found largely in plant materials, e.g. wheat germ, corn, sunflower seed, rapeseed, soybean oils, alfalfa and lettuce. Its complex biological functions may include antisterility, an antioxidant role, i.e. prevention of peroxide attack on unsaturated fatty acids in membrane lipids, and a cofactor between cytochrome *b* and *c* in the respiratory chain. The beneficial role of vitamin E

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in preventing degenerative physiological processes and ageing is still being investigated.

Although vitamin E intake in normal diets usually meets the daily requirement of children and adults, there is a constant commercial demand for this vitamin especially by elderly customers. Thus, the development of simple and sensitive methods other than the more expensive HPLC [1–3] and voltammetric [4] techniques for the determination of this vitamin in pharmaceutical preparations has always been a matter of concern.

The existing spectrophotometric methods for vitamin E determination make use of the oxidizability of the 6-hydroxychroman ring of α-tocopherol to the corresponding quinone, i.e., α-tocopherylquinone, by oxidizing agents finally giving coloured products. The official method utilizes the Fe(III)–bipyridyl [5] or bathophenanthroline [6] complex reagent as the oxidizing agent. The Fe(III) in these reagents is reduced by vitamin E to the corresponding Fe(II) complex, the absorbance of which is measured at 520 and 534 nm. Derivative spectrophotometric methods [7], which are not as specific as direct methods, have also been introduced.

In recent years, copper(II)–neocuproine reagent has been developed for determining a number of reducing agents in ammonium acetate-buffered solution [8], and biologically important reductants such as hydrogen peroxide, ascorbic acid and cysteine [9] have been assayed by the use of this reagent. This work was undertaken in order to establish the copper(II)–neocuproine spectrophotometric procedure as a standard method for analysing vitamin E solutions and pharmaceutical preparations.

α-Tocopheryl acetate, which is practically unaffected by the oxidizing influence of air, daylight and UV radiation, is more stable than the parent vitamin, α-tocopherol [10]. Since the acetate derivative is preferentially used in the formulation of pharmaceutical preparations, the conditions for the hydrolysis of this compound to α-tocopherol also need to be investigated for establishing a thorough assay method for vitamin E.

2. Experimental

2.1. Chemicals and reagents

α-Tocopheryl acetate was supplied by Roche (Turkey). Absolute ethanol and light petroleum (b.p. 60–71°C) were prepared from 95% technical alcohol and technical light petroleum (b.p. 60–80°C), respectively [11]. Bathophenanthroline was obtained from Sigma, technical alcohol from Turkish Monopolies, light petroleum from Delta Chemicals (Turkey) and nitrogen gas (bomb) from Habaş (Turkey). All remaining chemicals were supplied by Merck and were of analytical reagent grade. Rovigon and Supradyn dragées and Ephynal ampoules (Roche), the active ingredient of which is α-tocopheryl acetate, were freely purchased in the Turkish market.

2.2. Instrumentation

The molecular absorption spectra and absorbances at selected wavelengths were recorded with a Hitachi 220 A UV–visible spectrophotometer equipped with quartz cells of 1 cm light path. The pH of solutions was measured with a Metrohm E-512 pH meter using a glass electrode. The chromatograph was from Cecil Instruments (Cambridge, UK), and consisted of a pump (CE 1100 HPLC pump), a 20 μl injection valve (Model 7125, Rheodyne, Cotati, CA, USA), an analytical stainless-steel column packed with Spherisorb S5ODS1 (25 cm × 4.6 mm i.d.) from Hichrom (Berkshire, UK) and a variable-wavelength UV detector (CE 1220). Detection was effected at 292 nm for α-tocopherol. Chromatograms were obtained using an integrator (HP 3395 from Hewlett-Packard). Injections were made with a 25 μl syringe from Hamilton (Reno, NV, USA).

2.3. Solutions

α-Tocopherol solution was prepared at 2.46×10^{-4} M concentration in diethyl ether and absolute ethanol and diluted as necessary.

Neocuproine (2,9-dimethyl-1,10-phenanthroline) and bathophenanthroline (4,7-diphenyl-1,10-

phenanthroline) were prepared separately in ethanol and absolute ethanol, respectively, at a concentration of 3.0×10^{-3} M.

The pH 7.0 buffer was a 1.0 M solution of ammonium acetate in water. Solutions of 1.0×10^{-2} M $CuCl_2 \cdot 2H_2O$ and concentrated KOH (160 g per 100 ml) were also prepared separately in water. The Iron(III) chloride solution of concentration 2.0×10^{-3} M was prepared from $FeCl_3 \cdot 6H_2O$ in absolute ethanol. Phosphoric acid solution contained 0.172 M H_3PO_4 in absolute ethanol.

A light petroleum–absolute ethanol mixture was prepared by diluting 600 ml of light petroleum (b.p. 60–71°C) to 1.0 l with absolute ethanol.

The mobile phase for HPLC analysis was acetonitrile–chloroform–2-propanol–water (73:15:3.5:8.5, v/v).

2.4. Procedures

2.4.1. Hydrolysis of α-tocopheryl acetate to α-tocopherol [6]

A 1.10 g amount of α-tocopheryl acetate was dissolved in 4.5 ml of absolute ethanol and 0.33 g of ascorbic acid was added. The mixture was brought to boiling under a nitrogen atmosphere in a round-bottomed flask (equipped with a reflux condenser) immersed in a water-bath. A 1.1 ml volume of concentrated KOH solution was added and the mixture was refluxed for 15 min.

The flask was rapidly cooled, 25 ml of water were added and the mixture was extracted with three successive portions of 30 ml of diethyl ether. The ether phase was successively washed with water until the aqueous phase was neutral to phenolphthalein, dried with anhydrous sodium sulfate, filtered, concentrated to 5 ml under a nitrogen atmosphere by gentle heating and finally diluted to 10 ml with diethyl ether.

The purity and hydrolysis efficiency of the product were determined by comparison of the UV spectra of the hydrolysate and standard mixtures of α-tocopherol and α-tocopheryl acetate prepared in various proportions until the A_{292} of the hydrolysate matched that of a standard mixture. The hydrolysate was further subjected to HPLC analysis by the method of Kaplan et al. [1].

The hydrolysis conditions described above were optimized by changing one of the hydrolytic parameters while keeping the remaining three parameters constant, i.e. initial amount of α-tocopheryl acetate, volumes of absolute ethanol and concentrated KOH solution and amount of ascorbic acid. Since any remaining ascorbic acid from the hydrolysis procedure would interfere with the analysis of the reaction product (α-tocopherol) using the copper(II)–neocuproine reagent [8], the hyrolysis was repeated with a reagent blank not containing α-tocopheryl acetate.

2.4.2. Determination of α-tocopherol by the Fe(III)–bathophenanthroline method [6]

An aliquot of x ml ($0.2 \leqslant x \leqslant 0.4$ ml) of α-tocopherol in diethyl ether or preferentially in absolute ethanol was placed in a test-tube and $4.0 - x$ ml of light petroleum–ethanol and 1.0 ml of bathophenanthroline solution were added. The mixture was agitated, 0.5 ml of $FeCl_3$ solution was added dropwise and the solution was reagitated. After 15 s, 0.5 ml of H_3PO_4 solution was added. The absorbance of the solution at 534 nm against a reagent blank was measured after 3 min, and the colour was stable for 90 min. The procedure was repeated for α-tocopherol hydrolysed from the acetate derivative, and applied to the analysis of Rovigon and Supradyn dragées and Ephynal ampoules.

2.4.3. Determination of α-tocopherol by the Cu(II)–neocuproine method

In a test-tube were placed 1.0 ml of $CuCl_2$ solution, 2.5 ml of neocuproine solution, $3.0 - x$ ml of ethanol, 1.0 ml of ammonium acetate buffer and x ml ($0.3 \leqslant x \leqslant 0.6$ ml) of α-tocopherol solution (in diethyl ether or preferentially in absolute ethanol), in that order. The mixture was agitated and its absorbance was measured after 30 min at 450 nm against a reagent blank containing Cu(II)–neocuproine, solvent and buffer (the colour was stable for at least 90 more min). It is recommended to run three standards along with the sample for each determination. The procedure was applied to the analysis of the hydrolysis product, Rovigon and Supradyn dragées and Ephynal ampoules.

2.4.4. Preparation of pharmaceutical preparations for analysis

Eleven Rovigon dragées were finely ground in an agate mortar and five samples each containing approximately 110 mg of α-tocopheryl acetate were taken. Each of the samples was extracted with four successive portions of absolute ethanol and hydrolysed using the standard procedure.

The contents of eight Ephynal ampoules were thoroughly mixed and five samples each containing approximately 110 mg of α-tocopheryl acetate were taken and subsequently hydrolysed.

Three Supradyn dragées, each having a declared content of 10 mg of vitamin E equivalent, were finely ground, washed with 60 ml of distilled water and dried in an oven at 50°C. The residue was extracted with 30 ml of diethyl ether, evaporated and the residue dissolved in 10 ml of absolute ethanol. The ethanol solution was analysed by HPLC and hydrolysed using the standard hydrolysis procedure except for the amounts of ascorbic acid and KOH solution, which were chosen as 0.033 g and 0.03 ml, respectively, owing to the low vitamin E content. The ether solution of the hydrolysis product was diluted to 10 ml and diluted 20-fold with absolute ethanol such that the final solution contained 5% (v/v) of ether.

The spectra of the hydrolysis products (except Supradyn) were compared with that of standard α-tocopherol. The hydrolysis products were analysed by both spectrophotometric procedures and HPLC for α-tocopherol, and the original α-tocopheryl acetate contents of the pharmaceutical preparations were calculated by taking the efficiency factor of hydrolysis into consideration.

3. Results and discussion

The colour (i.e. A_{450} against the reagent blank) of the Cu(I)–neocuproine complex formed as a result of vitamin E oxidation stabilized after 30 min and remained the same for at least a further 90 min following complex formation.

The UV spectra of α-tocopherol and α-tocopheryl acetate (both 2.46×10^{-4} M) in ethanol is shown in Fig. 1. Since Beer's law was obeyed for both compounds at 292 nm within the concentration range of interest, the hydrolysis efficiency of the corresponding α-tocopheryl acetate solutions was estimated by comparing the absorbances at 292 nm of the hydrolysate and binary standard mixtures at this wavelength. This efficiency was found to be 93%, confirmed by HPLC, and was exploited in the analysis of pharmaceutical preparations of α-tocopheryl acetate.

In the hydrolysis procedure, half to twice as much α-tocopheryl acetate was not affected by an excess of KOH; e.g. 1.1 ml of concentrated KOH solution was sufficient to hydrolyse efficiently 2.20 g of α-tocopheryl acetate. KOH at levels up to 10 times the usual amount did not have a negative effect on hydrolysis.

It was also confirmed that ascorbic acid, introduced for the protection of α-tocopherol from oxidation during the hydrolysis of tocopheryl acetate, could be completely removed from the reaction product by thoroughly washing the organic (ether) phase with successive portions of water. Analysis of the hydrolysed α-tocopherol using the Cu(II)–neocuproine reagent revealed that not even a detectable amount of the ascorbic acid, which would normally show a positive interference in the method [8], accompanied the analyte.

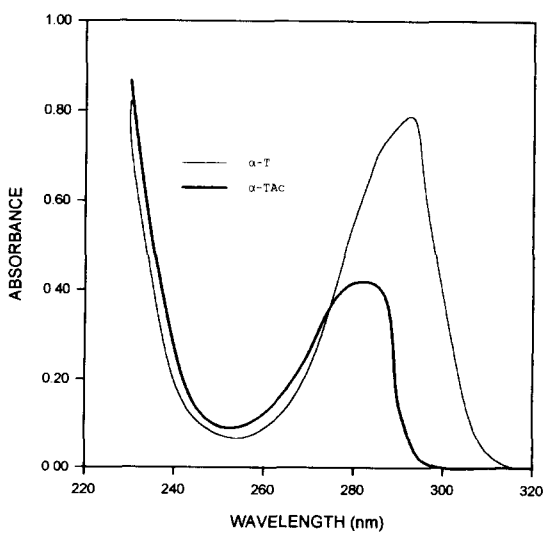

Fig. 1. UV spectrum of 2.46×10^{-4} M α-tocopherol and α-tocopheryl acetate in absolute ethanol.

The molar absorptivity, the concentration range for the validity of Beer's law and the precision of the developed method were found by the aid of absorbance measurements (at 450 nm) of standard α-tocopherol solutions. The spectrophotometric measurements were made on six-analyte sampling groups (whose absorbances (A_{450}) varied between 0.2 and 0.8) on three different occasions selected with 1 week time intervals. The within - and between-run precisions, also known as repeatability and reproducibility [12], respectively, were found by regression analysis of A_{450} vs. concentration curves, and expressed as the relative standard deviation (RSD, %) of the slope [13], i.e. of the molar absorptivity, of the absorbance vs. concentration regression lines.

The mean molar absorptivity was $\bar{\varepsilon} = 2.1 \times 10^4$ l mol^{-1} cm^{-1} with the RSD on each occasion being 2.3, 4.2 and 5.3% (repeatability). The overall linear equation relating the absorbance (A) and concentration (C in mol l^{-1}) of eighteen values at the 95% confidence level was

$$A = (2.1 \pm 0.1) \times 10^4 C + (0.68 \pm 1.7) \times 10^{-2}$$

with an RSD of the slope of 2.1% and a correlation coefficient of the linear plot $r = 0.996$ (reproducibility). Beer's law was obeyed between 2.4×10^{-6} and 9.0×10^{-5} M concentrations of α-tocopherol. The corresponding linear equation for calibration of the Fe(III)–bathophenanthroline method was found to be

$$A = (4.1 \pm 0.4) \times 10^4 C + (1.4 \pm 4.4) \times 10^{-2}$$

at the 95% confidence level with an RSD of 3.8% and $r = 0.996$. The latter method was slightly less reproducible and definitely more laborious (with respect to the preparation of reagents and solvents) than the developed method.

When the precisions of the developed Cu(II)–neocuproine and the standard HPLC method [1] using a slightly polarized mobile phase were compared (95% confidence level, 20 degrees of freedom), a pooled estimate of the standard deviation, s, may be calculated from the two individual standard deviations, s_1 and s_2, of the developed and reference methods (see Table 1):

$$s^2 = [(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2]/(n_1 + n_2 - 2) \quad (1)$$

Table 1
Comparison of the developed and reference methods for α-tocopherol determination

Sample number (n)	Amount of α-tocopherol found (μg)[a]	
	Cu(II)–neocuproine	HPLC
1	54.5	54.9
2	53.0	50.0
3	53.2	52.4
4	53.2	50.6
5	53.4	51.9
6	52.6	51.7
7	51.9	50.4
8	51.3	52.4
9	51.1	54.7
10	49.8	52.8
11	56.2	52.8
Standard deviation	$s_1 = 1.74$	$s_2 = 1.59$
Mean	$\bar{x}_1 = 52.7$	$\bar{x}_2 = 52.2$
Degrees of freedom	$n_1 + n_2 - 2 = 20$	
Critical t-value (95% confidence level)	2.09 [14]	

[a]Amount of α-tocopherol present = 53 μg.

$$t = (\bar{x}_1 + \bar{x}_2)/[s(1/n_1 + 1/n_2)^{1/2}] \quad (2)$$

where t has $n_1 + n_2 - 2 = 20$ degrees of freedom [14]. By applying Eqs. (1) and (2) to the data in Table 1, s was calculated as 1.67 and t was 0.70. Since this t value is smaller than the critical value, $t_{0.95}$, i.e. $0.70 < 2.09$, there is no significant difference between the developed and reference methods at the 95% confidence level, and the null hypothesis [14] is accepted.

Following the hydrolysis of α-tocopheryl acetate in commercial Rovigon and Supradyn dragées and Ephynal ampoules, inspection of the hydrolysates by UV and HPLC (only the latter applicable for Supradyn) confirmed that the hydrolysis product was essentially α-tocopherol. The analyses of these commercial preparations by HPLC and the two spectrophotometric methods are depicted in Table 2. Five samples of each preparation were analysed. The results confirm that the developed method is not inferior in precision to the literature spectrophotometric and HPLC methods, even in a complex multivitamin

Table 2
Comparison of analysis results of commercial formulations of vitamin E (after hydrolysis)

Sample[a]	Form	α-tocopheryl acetate declared (mg)	Amount of vitamin E found (mg)		
			HPLC	Cu(II)–neocuproine	Fe(III) –bathophenanthroline
Rovigon	Dragée	70	65 ± 2	66 ± 2	66 ± 3
Ephynal	Ampoule	100	102 ± 3	103 ± 3	103 ± 4
Supradyn	Dragée	30[b]	21 ± 1	23 ± 2	20 ± 2

[a] All from Roche.
[b] Amount of α-tocopheryl acetate before alkaline hydrolysis was found to be 22 ± 1 mg by HPLC.

and mineral combination such as Supradyn (containing 11 essential vitamins, five minerals comprising the reducing $FeSO_4$ and three oligo-elements of Cu(II), Zn(II) and Mo(VI)).

Since the observed molar absorptivity of the Fe(III)–bathophenanthroline method for vitamin E is approximately twice that of the literature value for this complex reagent [6,15], it may be deduced that vitamin E undergoes a two-electron oxidation with this reagent, possibly to α-tocopherylquinone [16]. In the original paper introducing the copper(II)–neocuproin reagent [8], the authors showed that a two-electron oxidation of a reducing analyte would normally be expected to exhibit a mean molar absorptivity of 1.6×10^4, although higher values were encountered such as those of glutathione ($\varepsilon = 8.5 \times 10^3$, le oxidation) and hydroxylamine ($\varepsilon = 1.8 \times 10^4$, 2e oxidation), depending on the nature of the analyte [8], and solvent effects may also cause some enhancement [17] owing to the affinity of the $[Cu\text{-neocuproine})_2]^+$ cation, i.e. the reduction product of Cu(II)–neocuproine, to add a solvent molecule as ligand in the charge-transfer excited state, thereby increasing the molar absorptivity to a certain extent. When the standard reduction potentials of the two complex reagents are considered, the $[Cu(neocuproine)_2]^{2+,1+}$ couple has been reported to exhibit a potential of $E_c^\circ = 0.603$ V [17–19], while the oxidizing power of Fe(III)–bathophenanthroline is considerably weaker than that of Fe(III)–phenanthroline ($E_c^\circ = 1.2$ V), which is reflected in the observation that Fe(III)–bathophenanthroline is not as easily reduced as the Fe(III)–phenanthroline analogue [20]. The Fe(III)–bathophenanthroline reagent should be an even less potent oxidant in the presence of phosphoric acid (as described in Section 2.4), because it is known that the 0.77 V standard potential of the uncomplexed $Fe^{3+,2+}$ redox couple is reduced to 0.44 V in 0.3 M H_3PO_4 medium [21]. Thus, both the Cu(II)–neocuproine and Fe(III)–bathophenanthroline reagents are oxidizing agents of comparable strength under the experimental conditions employed, and it is reasonable to assume that both reagents lead to 2e oxidation of α-tocopherol as accomplished with other oxidizing reagents such as tris(2,2′-bipyridyl)iron(III), $FeCl_3$ and $Fe(CN)_6^{3-}$ [16].

Although the molar absorptivity of the Fe(III)–bathophenanthroline method is greater than that of the developed method, allowing the achievement of greater sensitivity with the former, the Cu(II)–neocuproine method is less laborious and shows the potential to work better in complex formulations. Moreover, the introduced reagent performs its action in a neutral medium, i.e. ammonium acetate-buffered solution of pH 7.0, where some common potential interferents such as glucose (and reducing sugars with the —$(CHOH)_n$—CHO group) and oxalate would not be oxidized [8], unlike in acidic media where most oxidants should function, thereby adding some selectivity to the proposed method. As vitamin E is not soluble in water, pharmaceutically common interferents which are reducing agents, e.g. ascorbic acid and iron(II) sulfate, would also not interfer after a preliminary aqueous extraction stage. The method is capable of differentiating α-tocopherol from the pharmaceutically desired ana-

logue, α-tocopheryl acetate, where the latter may respond to the method only after hydrolysis.

Both the developed and alternative spectrophotometric methods have the advantage of working in the visible range, permitting the use of simple colorimeters where required instead of the more expensive spectrophotometers. The developed method has a slight superiority in that all solutions of this method except neocuproine are prepared in water while the Fe(III)–bathophenanthroline method requires the preparation of absolute EtOH and a certain boiling range fraction of light petroleum. Both methods have similar precision and, in addition, the Cu(II)–neocuproine reagent in a routine analytical laboratory, lacking more expensive HPLC and GC instruments, handling other reducing substances [8,9] would be more versatile.

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