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Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 33–38
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Direct high-performance liquid chromatographic analysis of D-tocopheryl acid succinate and derivatives

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Received 9 November 2004; received in revised form 17 February 2005; accepted 18 February 2005
Available online 13 April 2005

Abstract

A method of analysis of a Vitamin E derivative D-tocopheryl acid succinate (TS) in biological fluids and commercially available products is necessary to study the kinetics of in vitro and in vivo metabolism, tissue distribution, and content uniformity. A simple and inexpensive high-performance liquid chromatographic method was developed for the direct determination of D-tocopheryl acid succinate in commercially available products, rat serum, and rat tissues. This method can also be applied to the determination of 15 Vitamin E derivatives. Rat serum (0.1 ml) was extracted with sodium dodecyl sulfate, ethanol, hexane, and then dried under nitrogen gas after addition of the internal standard, DL-α-tocopherol acetate. Separation was achieved on a C18 column with UV detection at 205 nm. The calibration curve for D-tocopheryl acid succinate was linear ranging from 0.025 to 100 μg/ml. The mean extraction efficiency was >92%. Precision of the assay was <5% (CV), and was within 5% at the limit of quantitation (0.025 μg/ml). Bias of the assay was lower than 5%, and was within 5% at the limit of quantitation. The assay was applied successfully to the serum and tissue distribution of D-tocopheryl acid succinate in rats, various Vitamin E derivatives, and content uniformity in commercially available products containing D-tocopheryl acid succinate.

Keywords: Reversed-phase HPLC; UV-detection; Vitamin E; Tocopherol; D-Tocopheryl acid succinate

1. Introduction

D-α-Tocopherol hydrogen succinate, also referred to as D-tocopheryl acid succinate (TS), is a derivative of Vitamin E. It has anti-oxidant properties and is being examined experimentally for possible anti-cancer effects while being commercially available as an over the counter medication [1,2]. We have previously published a sensitive, indirect, high-performance liquid chromatography (HPLC) method using fluorometric detection for the simultaneous determination of the non-hydrolysable tocopherol succinate ether analog, D-α-tocopheroloxybutyric acid (TSE) and α-tocopherol in biological specimens using fluorescence. Using this method, TS can be indirectly quantified after hydrolysis of the succinate moiety as tocopherol [3].

It is probable that the pharmacokinetic disposition of TS plays a critical role in the activity of TS, and its unique protective abilities are related to the tissue distribution of TS. The pharmacokinetic basis for the action and disposition of TS has not been delineated and no pharmacokinetic studies have previously been published in any species after the parenteral administration. Interestingly, neither the United States government nor any other agency is routinely testing D-tocopheryl acid succinate supplements for their contents or quality. The lack of this basic pharmaceutical information of TS is in part due to a deficit of a simple validated and direct method of analysis of TS available in the literature.

To our knowledge, no study has been published characterizing the separation of D-tocopheryl acid succinate in pharma-

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doi:10.1016/j.jpba.2005.02.029

cokinetic studies, as there are no validated direct methods of analysis of D-tocopheryl acid succinate evaluating commercially available products in the literature. There is, however, a single abstract suggesting that the determination of TS is possible in human plasma although assay details are incomplete, validation is lacking and tocopherol was not able to be simultaneously quantified [4]. The present study describes a simple, direct, isocratic, reversed-phase high-performance liquid chromatography (HPLC) method for the determination of D-tocopheryl acid succinate and its application to rat samples and content uniformity studies.

2. Experimental

2.1. Chemicals and reagents

D-Tocopheryl acid succinate, potassium hydroxide, DL-α-tocopherol acetate, and tocopherol nicotinate were purchased from Sigma Chemicals (St. Louis, MO, USA). HPLC grade acetonitrile, 12 M hydrochloric acid, and HPLC water were purchased from J.T. Baker (Phillipsburg, NJ, USA). D-α-Tocopherol polyethylene glycol 1000 succinate was a gift from Eastman Chemical Company (Kingsport, TN, USA). L-Ascorbic acid, ethanol, hexane, and HPLC grade methanol were purchased from Fisher (Fair Lawn, NJ, USA). Sodium dodecyl sulfate (SDS) was purchased from Gibco BRL (Grand Island, NY, USA). γ-Tocopherol succinate, α-tocopherol 3-methyl succinate, α-tocopherol 2,2-dimethyl glutarate, α-tocopherol glutamic acid, α-tocopherol 2,2-dimethyl succinate tris salt, δ-tocopherol succinate tris, γ-tocopherol succinate-tris, D-alpha-tocopheroloxybutyric free-acid, α-tocopherol 2-methyl succinate and δ-tocopherol were synthesized by Dr. Doyle Smith, Virginia Commonwealth University and provided by Dr. Fariss's Laboratory (UCHSC, Denver, CO). D-α Tocopherol, and D-γ-tocopherol and D-δ-tocopherol were obtained from Cognis (LaGrange, Il, USA). D-Tocopheryl acid succinate tablets (Bronson Natural Vitamin E 300 IU® Lindon, UT, USA) were purchased on the open market through an on-line retailer. In addition to D-tocopheryl acid succinate, the composition of these tablets include cellulose, stearic acid, silica, calcium silicate, sodium croscarmellose, talc, magnesium stearate, hydroxypropyl methylcellulose, maltodextrin, polyethylene glycol, lechtin and sodium citrate. Rats were obtained from Charles River Laboratories (Wilmington, MA, USA). Ethics approval for animal experiments was obtained from Washington State University (Pullman, WA USA).

2.2. Chromatographic system and conditions

The HPLC system utilized was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10AT VP pump, a SIL-10AF auto-injector, an SPD-M10A VP spectrophotometric diodearray detector, and an SCL-10A VP system controller. Data collection and integration were accomplished using Shimadzu EZ Start 7.1.1 SP1 software. The analytical column used was a (Jones chromatography) C18 column (3.3 cm × 4.6 mm i.d., 3-μm particle size, Lakewood, CO, USA). The mobile phase consisted of acetonitrile and water (90:10, v/v), filtered and degassed under reduced pressure, prior to use. Separation was carried out isocratically at ambient temperature (25 ± 1 °C), and a flow rate of 1.3 mL/min, with ultraviolet (UV) detection at 205 nm.

2.3. Stock and working standard solutions

Ten milligrams of D-tocopheryl acid succinate was accurately weighed on an analytical balance AG245, Mettler (Toledo, OH, USA) and dissolved with methanol in a 100-mL volumetric flask to make a stock standard solution in methanol with a concentration of 100 μg/mL. A methanolic stock solution of α-tocopherol acetate (internal standard) was prepared similarly with a concentration of 100 μg/mL. These solutions were protected from light and stored at −20 °C between uses, for no longer than 3 months. Calibration standards in serum were prepared daily from the stock solution of D-tocopheryl acid succinate by sequential dilution with blank rat serum yielding a series of concentrations namely, 0.025, 0.05, 0.1, 1.0, 10.0, and 100.0 μg/mL of each in the three replicates.

Quality control (QC) samples were prepared (in a blinded fashion) from the stock solution of D-tocopheryl acid succinate by dilution with blank biological fluid to yield target concentrations of 0.025, 10.0, and 100.0 μg/ml. The QC samples were prepared independently from the calibration standards and divided into 0.1 mL aliquots in micro centrifuge tubes, and stored at −70 °C before use.

2.4. Sample preparation

The working standards (50 μL), samples (15–25 μg for tissues or 100 μL for serum), and 50 μL of tocopherol acetate internal standard solution (μg/ml) were all added to a 1.9 mL polypropylene tube. Sodium dodecyl sulfate (SDS 0.3 mL of 100 mM)/1% L-ascorbic acid in water, 0.45 mL ethanol, and 0.8 mL of hexane were added to the samples tubes. The solution was vortexed briefly after the addition of every solvent until the addition of hexane, after which the tubes were vortexed for 30 s (Vortex Genie-2, VWR Scientific, West Chester, PA, USA), and then centrifuged at 13,000 rpm for 2 min (Beckman Microfuge centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). The hexane layer was aspirated, added to a new 1.9 mL tube, and dried down under a stream of nitrogen. The working standards and samples were subsequently reconstituted in a volume ranging from 0.3 to 1.0 mL in a 2.5% methanol/ascorbic acid solution, depending on the projected tissue or serum concentrations, and 150 μL of the final product was injected into the HPLC system. The 2.5% methanol/ascorbic acid solution was made by dissolving 2.5 g ascorbic acid in 100 mL methanol. Five tablets containing D-tocopheryl acid succinate were weighed and trans-

ferred to a mortar. They were ground and three portions of the resulting powder, each equivalent to 10% of the total weight, were dissolved in 1 L of water. The resultant was filtered, and 1 mL of the filtrate was diluted to 2.5 mL with water. α-Tocopherol acetate (internal standard) was added to 0.05 mL of this solution. The mixture was evaporated to dryness and reconstituted in 0.2 mL mobile phase. A 150 μL (v/v) of the eluted solution was injected into an isocratic HPLC system.

Approximately, 0.5 mg of each of the 15 TS derivatives, listed in Section 2.1, were dissolved in 0.2 mL mobile phase, and 150 μL of each solution was injected into the isocratic HPLC system mentioned previously.

2.5. Precision and accuracy

The within-run precision and accuracy of the replicate assays ($n = 6$) were tested by using six different concentrations of D-tocopheryl acid succinate, namely 0.025, 0.05, 0.1, 1.0, 10.0, and 100.0 μg/mL. The between-run precision and accuracy of the assays were estimated from the results of six replicate assays of QC samples on six different days within 1 week. The precision was evaluated by the relative standard deviation (R.S.D.). The accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration [5].

2.6. Recovery

Recovery for D-tocopheryl acid succinate from biological fluids was assessed ($n = 6$) at 0.025, 0.05, 0.1, 1.0, 10.0, and 100.0 μg/mL and the recovery of the internal standard was evaluated at the concentration used in sample analysis (25 μg/mL). A known amount of D-tocopheryl acid succinate was spiked into 0.1 ml biological fluid to give the above concentrations. The samples were treated as described under Section 2.4, and analyzed by HPLC. The extraction efficiency was determined by comparing the peak areas of D-tocopheryl acid succinate to those of D-tocopheryl acid succinate or solutions of corresponding concentration injected directly in the HPLC system without extraction. The mean recovery of D-tocopheryl acid succinate from rat serum varied from 92.2 to 95.7 (Table 2).

2.7. Freeze–thaw and bench-top stability of D-tocopheryl acid succinate samples

The freeze–thaw stability of D-tocopheryl acid succinate was evaluated at 0.025, 10.0, and 100.0 μg/mL, using QC samples. These samples were analyzed in triplicate without being frozen at first, and then stored at −70 °C and thawed at room temperature (25 ± 1 °C) for three cycles.

The stability of D-tocopheryl acid succinate in reconstituted extracts during run-time in the HPLC auto-injector was investigated using the pooled extracts from QC samples of three concentration levels 0.025, 10.0, and 100.0 μg/mL. Samples were kept in the sample rack of the auto-injector and injected into HPLC system every 4 h, from 0 to 24 h at the temperature of auto-injector (26 ± 1 °C).

Table 1
Tissue concentrations of T and TS, in PEG-400 after 48-h post-IV dose administration in rats ($n = 4$, μg/g, mean ± S.D.)

Tissue	TS and T 48 h	
	TS	T
Brain	0.003 ± 0.001	0.016 ± 0.003
Heart	0.071 ± 0.025	0.038 ± 0.001
Lung	0.218 ± 0.045	0.060 ± 0.006
Liver	0.625 ± 0.095	0.079 ± 0.010
Kidney	0.032 ± 0.008	0.028 ± 0.001

2.8. Pharmacokinetics of TS in rat

Male Sprague–Dawley rats (200–250 g) were anaesthetized using halothane and a silastic catheter was cannulated into the right jugular vein. Animals were placed in metabolic cages, allowed to recover overnight and fasted for 12 h before dosing. On the day of experiments, animals were dosed i.v. with TS (100 mg/kg) in polyethylene glycol 400. Serial blood samples (0.25 ml) were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8, and 12 and 24 h. After each sample collection, the cannula was flushed with 0.25 ml of saline. Following centrifugation of the blood samples, the serum was transferred to a new polypropylene tube and stored at −70 °C until analyzed using the current method for TS and a previous method for tocopherol [3].

At 48-h post-dose, animals were anaesthetized with sodium pentobarbital (60 mg/mL) via i.p. injection. Whole blood was removed via a cardiac puncture using an 18-gauge needle attached to a 5 mL syringe. Tissues were removed and homogenized for analysis, as previously described (Table 1) [5].

2.8.1. Pharmacokinetic analysis

The elimination rate constant (λ_n) was estimated by linear regression of the serum concentrations in the log-linear terminal phase. In order to estimate serum concentrations (C_0) immediately after i.v. injection of TS, compartmental models were fitted to the serum concentration versus time data using WinNonlin® (version 1.0). The estimated C_0 was then used in conjunction with the actual measured serum concentrations to determine the area under the serum concentration–time curve (AUC). The $AUC_{0-\infty}$ was calculated using the combined log-linear trapezoidal rule for data from time of dosing to the last measured concentration, plus the quotient of the last measured concentration divided by λ_n. Non-compartmental pharmacokinetic methods were used to calculate clearance (CL) and volume of distribution (V_d) after i.v. dosing.

2.8.2. Statistical analysis

Statistical analysis of the pharmacokinetic data was performed using Student's *t*-test for unpaired samples. A *p*-value of less than 0.05 was considered statistically significant. Sta-

tistical analysis was performed using Microsoft Excel 97 (Microsoft, Redmond, WA). Summary data were expressed as mean ± standard deviation (S.D.).

2.9. Data analysis

Quantification was based on calibration curves constructed using peak area ratio (PAR) of D-tocopheryl acid succinate to internal standard, against D-tocopheryl acid succinate concentrations using unweighted least squares linear regression.

3. Results and discussion

3.1. Chromatography

Separation of D-tocopheryl acid succinate and the internal standard in biological fluids was achieved successfully. There were no interfering peaks co-eluted with the compounds of interest (Fig. 1). The retention time of D-tocopheryl acid succinate was approximately 12 min (Fig. 2). The internal standard was eluted at approximately 28 min. Using this same mobile phase composition and consitions, 15 other tocopherol and tocopherol succinate derivatives could also be readily detected (Table 2 and Fig. 3).

The performance of the HPLC assay was assessed using the following parameters, namely: peak shape and purity, interference from endogenous substances in biological fluid, linearity, limit of quantitation (LOQ), freeze–thaw stability, stability of reconstituted extracts, precision, accuracy, and recovery. Various compositions of mobile phase were tested to achieve the best resolution of D-tocopheryl acid succinate.

There are no other validated assays directly quantifying D-tocopheryl acid succinate in biological matrices published in the literature. The present assay is practical to use in preclinical applications of D-tocopheryl acid succinate where small sample volumes are obtained and has been cross-validated to human plasma samples (unpublished observations).

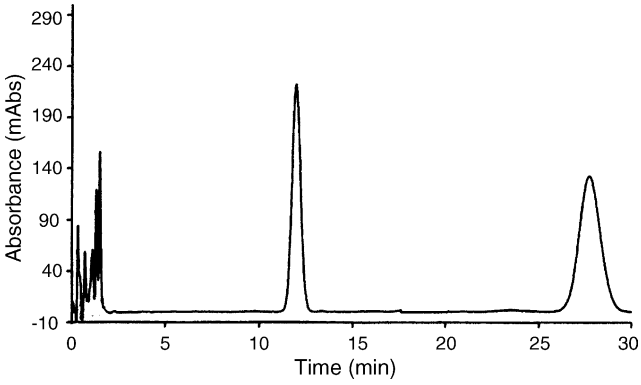

Fig. 1. Representative chromatogram of rat serum containing D-tocopheryl acid succinate (100 μg/ml) and the internal standard (IS) 25 μg/ml.

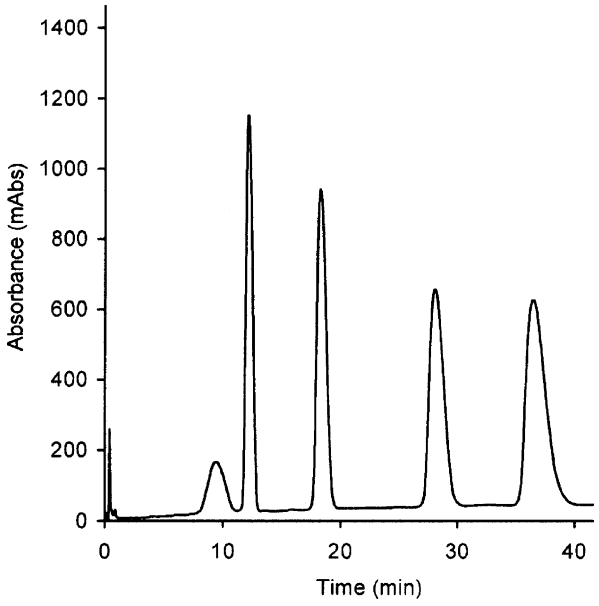

Fig. 2. Chromatogram of TS derivatives: TSPEG, α-tocopherol succinate, α-tocopherol, tocopherol acetate, and α-tocopherol nicotinate, in respective elution order.

Table 2
Tocopherol succinate derivatives and retention times (min)

δ-Tocopheryl succinate	9.25
D-α-Tocopheryl polyethylene glycol 1000 succinate	9.26
δ-Tocopherol succinate	9.80
γ-Tocopherol succinate	11.00
α-Tocopherol succinate	12.00
α-Tocopherol glutarate	14.32
α-Tocopherol 2-methyl succinate	14.84
α-Tocopherol 3-methyl succinate	15.50
α-Tocopherol 2,2-dimethyl glutarate	18.52
α-Tocopherol	18.66
α-Tocopherol 2,2-dimethyl succinate	19.04
D-α-Tocopheryloxybutyric acid	19.05
α-Tocopherol acetate	28.03
α-Tocopheryl nicotinate	36.48

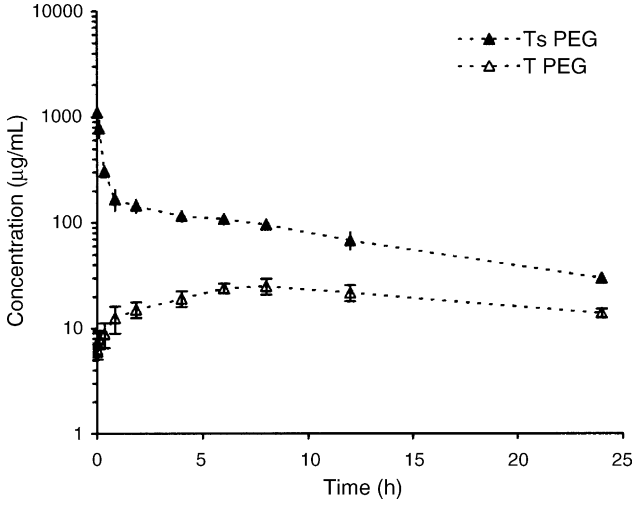

Fig. 3. Serum concentrations of D-tocopheryl acid succinate 100 mg/kg, following the i.v. administration to rats ($n = 4$, mean ± S.D.).

Table 3
Within- and between-day precision and accuracy of the assay for Vitamin E succinate (TS) in rat plasma

Concentration (μg/ml)	Within-day ($n = 6$)			Between-day ($\alpha = 6$)		
Actual	Observed	RSD (%)	Bias (%)	Observed	RSD (%)	Bias (%)
0.025	0.024	4.5	−5.0	0.026	4.1	−3.9
0.05	0.049	3.6	−1.2	0.05	4.0	1.3
0.1	0.1	4.5	1.9	0.11	6.3	4.8
1	1.01	4.3	−4.7	1.02	5.8	−2.9
10	9.76	1.8	−3.8	10.00	3.9	1.4
100	101.0	2.4	2.1	100.3	2.6	2.2

3.2. Linearity and LOQ

Excellent linear relationships ($r^2 = 1$) were demonstrated between peak area ratio (PAR) of D-tocopheryl acid succinate to the internal standard and the corresponding serum concentrations of D-tocopheryl acid succinate over a range of 0.025–100 μg/mL. The mean regression-line from the validation runs was described by D-tocopheryl acid succinate (μg/mL) = $0.0074x + 0.0033$.

The LOQ of this assay was 25.0 ng/mL in biological fluids, with the corresponding between day relative standard deviation of 5.12% for D-tocopheryl acid succinate and a bias of 4.55%.

3.3. Precision, accuracy and recovery

The within- and between-run precision (R.S.D.) calculated during replicate assays ($n = 6$) of D-tocopheryl acid succinate in rat serum was <5% over a wide range of concentrations (Table 3). The intra- and inter-run bias assessed during the replicate assays for D-tocopheryl acid succinate varied between 1.3 and 5.0% (Table 3). These data indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for D-tocopheryl acid succinate from biological fluids varied from 92.2 to 95.7%. In addition, the recovery of tocopherol acetate was 94.1% at its concentration used in the assay. High recovery from biological fluids suggested that there was negligible loss of D-tocopheryl acid succinate and tocopherol acetate during the extraction process. Additionally, the efficiencies of extraction of D-tocopheryl acid succinate and α-tocopherol acetate were comparable.

3.4. Stability of D-tocopheryl acid succinate samples

No significant degradation was detected after the samples of D-tocopheryl acid succinate in biological fluids following three freeze-thaw circles. The recoveries of D-tocopheryl acid succinate were, respectively, from 98.5 to 99.7%, following three freeze-thaw cycles for D-tocopheryl acid succinate QC samples of D-tocopheryl acid succinate or α-tocopherol acetate. There was no significant decomposition observed after the reconstituted extracts of D-tocopheryl acid succinate were stored in the auto-injector at room temperature for 24 h. The measurements were from 98.2 to 99.9% of the initial value for extracts of D-tocopheryl acid succinate in biological fluids of 0.025, 1.0, and 100.0 μg/mL, respectively, during the storage in the auto-injector at room temperature for 24 h.

3.5. Pharmacokinetics and tissue distribution in rat

The HPLC method has been applied to the determination of D-tocopheryl acid succinate disposition in a rat. (Fig. 4). Distribution of D-tocopheryl acid succinate into tissues was apparent, with the highest concentrations of D-tocopheryl acid succinate in liver and lung. As expected, baseline endogenous concentrations of tocopherol were present in both groups, with a net increase in tocopherol levels, occurring as D-tocopheryl acid succinate was metabolized to tocopherol. The tocopherol concentration slowly peaked in serum between 7- and 8-h post-dose, and exhibited a low clearance rate. Intravenous D-tocopheryl acid succinate administration also resulted in significant tocopherol distribution in all tissues examined, which was especially abundant in the liver and lung. These results suggest that D-tocopheryl acid succinate may be useful for the delivery of tocopherol and D-tocopheryl acid succinate to the lung and liver for anti-oxidant and potential anti-cancer activity [6].

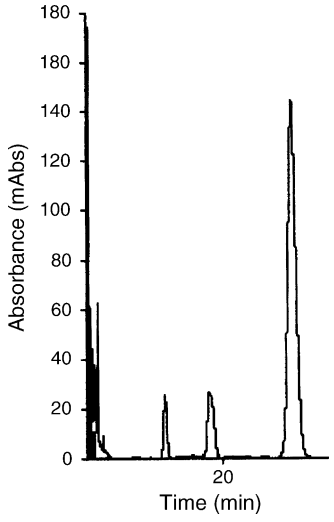

Fig. 4. Chromatogram of 100 mg/kg IV dose of TS in rat serum after 12 h; compounds in respective elution order are α-tocopherol succinate, α-tocopherol and IS, tocopherol acetate.

3.6. Content of D-tocopheryl acid succinate in tablet

The HPLC method has been applied to the determination of D-tocopheryl acid succinate in a commercially available tablet. Five tablets containing D-tocopheryl acid succinate were weighed and transferred to a mortar. They were ground and three portions of the resulting powder, each equivalent to 10% of the total weight, were dissolved in 1 L of water. The resultant was filtered and 1 mL of the filtrate was diluted to 2.5 mL with water. α-Tocopherol acetate (internal standard) was added to 0.05 mL of this solution. The mixture was evaporated to dryness and reconstituted in 0.2 mL mobile phase. A 0.1 mL volume of the eluted solution was injected into an isocratic HPLC system. The average content of five tablets analyzed was $104 \pm 3\%$.

In summary, the developed HPLC assay method is direct, reproducible, simple and accurate. It has been successfully applied to a disposition study of D-tocopheryl acid succinate in rats, a commercially available D-tocopheryl acid succinate tablet, and to the detection of various TS derivatives.

Acknowledgements

Vitamin Settlement Research Grant Attorney General's Office, State of Washington Optimizing Vitamin E Protection in the Diabetic Population and National Institutes of Health. R21 Grant and a Summer Undergraduate Research Fellowship to R.L.G.

References

[1] J. Neuzil, M. Tomasetti, A.S. Mellick, R. Alleva, B.A. Salvatore, M. Birringer, M.W. Fariss, Curr. Cancer Drug Targets 4 (2004) 355–372.
[2] K.N. Prasad, B. Kumar, X.D. Yan, A.J. Hanson, W.C. Cole, J. Am. Coll. Nutr. 22 (2003) 108–117.
[3] M.A. Tirmenstein, B.W. Watson, N.C. Haar, M.W. Fariss, J. Chromatogr. B: Biomed. Sci. Appl. 707 (1998) 308–311.
[4] P.H. Anderson, H. Shi, M. Malafa, AAPS Pharm. Sci. 4 (2003) 259.
[5] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yocobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittaman, S. Spector, Int. J. Pharm. 82 (1992) 1–7.
[6] R. Woody, C. Fukuda, K.A. Roupe, M.W. Fariss, G.D. Clifton, N.M. Davies, J. Pharm. Pharm. Sci. 7 (2004) A72.