Determination of Vitamin E Acid Succinate in Biodegradable Microspheres by Reversed-Phase High-Performance Liquid Chromatography

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Abstract

A simple, rapid, and reproducible reversed-phase high-performance liquid chromatographic (HPLC) method is applied to the routine assay of vitamin E acid succinate in biodegradable microspheres. Vitamin E acid-succinate-containing poly-(D,L-lactic-co-glycolic acid) microspheres are prepared by the solvent evaporation method. The starting drug–polymer ratio is 1:10 (w/w) and the total amount of drug and polymer processed is always 440 mg. The content of vitamin E acid succinate in the microspheres is evaluated by HPLC. Chromatography is carried out isocratically at 25°C ± 0.5°C on an Extrasil ODS-2 column with a mobile phase composed of methanol–water (97:3, v/v) (pH 5.6) at a flow rate of 2 mL/min and UV detection at 284 nm. Parameters such as linearity, limits of quantitation (LOQ) and detection (LOD), precision, accuracy, recovery, specificity, and ruggedness are studied as reported in the International Conference on Harmonization guidelines. The stability of vitamin E acid succinate is also studied with satisfactory results after 48 h at 25°C. The method is selective and linear for drug concentrations in the range 15–210 µg/mL. The LOQ and LOD are 15 and 3 µg/mL, respectively. The results for accuracy studies are good. Values for coefficient of variation for intra- and interassay are 2.08% and 2.32%, respectively. The mean percentage of vitamin E acid succinate in the recovery studies is 99.52% ± 0.81%. The mean loading efficiency for microspheres is 96.53% ± 1.31%.

Introduction

Proliferative vitreoretinopathy (PVR) is the most common cause of failure after vitreoretinal surgery, leading to traction retinal detachment after a previous successful surgical outcome. Although several types of drugs have been used to prevent PVR development, most of them have shown retinal toxicity. Vitamin E (α-tocopherol) is a physiologic retinal compound that plays an important role as a retinal antioxidant. Previous studies assessed an inhibitory effect of vitamin E on fibroblast and retinal pigment epithelium cell proliferation in vitro. These cell types play a major role in PVR development. Vitamin E acid succinate has also been reported to share the antiproliferative properties of vitamin E in vitro. When vitamin E and vitamin E acid succinate were administered in solution to experimental animals, both decreased the development of PVR during the first days of treatment, without retinal toxicity, but vitamin E acid succinate showed better antiproliferative activity than vitamin E (1).

For this reason, the release of vitamin E acid succinate from a biodegradable drug delivery system such as microspheres, with the capability to promote prolonged release of the drug, may help to create conditions to prevent the later development of PVR (2), and this offers a good alternative to multiple administrations. The advantage of these erodible devices is that they degrade and disappear from the site of implantation when a biodegradable and biocompatible polymer is employed as the microparticle carrier. Poly-(D,L-lactic-co-glycolic acid) (PLGA) is a common biodegradable medical polymer with a history of safe human usage in sutures, orthopaedics, bone plates, and extended-release pharmaceuticals (3). Biological degradation of PLGA occurs primarily by hydrolysis, and the degradation products are lactic and glycolic acids, which are further metabolized to carbon dioxide and water (4).

The determination of drug content in biodegradable microspheres (loading efficiency) is important because the dose of microparticles to be administered depends on it. Although vitamin E acid succinate shows a maximum absorbance at 284 nm, spectrophotometry is not suitable to determine loading efficiency because the polymer employed in making the microspheres interferes with the drug.

Several analytical techniques have been employed for the determination of vitamin E. Procedures such as spectrophotometry, fluorimetry, or polarography require isolation and purification steps to remove interfering compounds. Other procedures for tocopherols and vitamin E acid succinate are based on gas chromatography, but these assays are rather lengthy. Many high-performance liquid chromatographic (HPLC) methods for single or simultaneous multiple measurements using UV–vis absorbance

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or electrochemical detection have been reported to determine vitamin E (5–11) and its derivatives, vitamin E acetate (12–17) and vitamin E nicotinate (18), in biological samples, pharmaceutical preparations, cosmetics, and foods. These methods of analysis allow the quantitation of vitamin E and its derivatives at very low limits of detection (LOD), but selectivity and sensitivity of vitamin E determinations can be, without a doubt, effectively improved by the use of several approaches such as double- instead of single-column separation, gradient instead of isocratic elution, two or three separate HPLC lines connected to different detectors, or even three to four types of solvents for the mobile phase. The complexity of these HPLC conditions makes these methods complicated and costly for routine analysis (19). Furthermore, it must be pointed out that none of the mentioned HPLC methods have been reported for vitamin E acid succinate. Thus, the objective of this work was the development of a rapid and simple HPLC method with UV detection for quantitation of vitamin E acid succinate in biodegradable microspheres.

Experimental

Materials

Vitamin E acid succinate [2,5,7,8-tetramethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol acid succinate] was purchased from Sigma Chemical Co. (Madrid, Spain). HPLC-grade methanol was supplied by Lab-Scan (Dublin, Ireland), and acetic acid of analytical grade was provided by Merck (Barcelona, Spain).

Poly-D,L-lactic-co-glycolic acid (50:50) (inherent viscosity 0.2 dL/g) was purchased from Boehringer Ingelheim Chemicals Division (Ingelheim, Germany). Polyvinyl alcohol (PVA) MW 72000 Dalton (Fluka Chemie AG, Germany) and vitamin E (α-tocopherol) (Sigma Chemical Co.) were also used. Methylene chloride (CH_2Cl_2) and HPLC-grade ethanol were obtained from Merck. Demineralized water was purified in a Milli-Q filtration system (Millipore, Bedford, MA) to obtain HPLC-grade water.

Microparticle preparation

Microparticle preparation was performed by the solvent evaporation technique from an oil-in-water emulsion (20,21). The starting drug–polymer ratio was 1:10 (w/w), with 400 mg being the amount of polymer processed. Briefly, the organic phase was prepared by dissolving 400 mg of PLGA in 1 mL CH_2Cl_2 using a vortex mixer (IKA Labortechnik, Staufen, Germany) and then dispersing an equivalent to 40 mg of vitamin E acid succinate in the polymer solution. The aqueous phase consisted of a PVA solution (0.1%). The organic phase containing the drug was slowly added to the aqueous phase to form an emulsion, and the system was continuously stirred for 3 h at room temperature to allow complete evaporation of the organic solvent.

After evaporation of the organic solvent, the microspheres were vacuum-filtered through a 5-µm membrane filter, washed three times with distilled water, and freeze-dried (FTS Systems, Stone Ridge, NY). The freeze-dried microspheres were kept in a dessicator until use. All processes were performed with minimum exposure of the samples to light using dark or aluminium foil-wrapped containers. Microspheres without vitamin E acid succinate were also prepared according to the related technique to carry out the recovery studies.

Apparatus and chromatographic conditions

Chromatographic analyses were carried out using a Gilson HPLC instrument (Middleton, WI), consisting of a model 305 pump, a model 118 UV–vis detector, and a model 712 system controller software. The injector was equipped with a 20-µL loop (model 7125, Rheodyne, Berkeley, CA). The chromatographic separation was performed on an Extrasil ODS-2 analytical column (250- × 4.0-mm i.d., 5-µm particle size) directly connected to an octadecyl siloxane guard column, both purchased from Teknokroma (Barcelona, Spain). The mobile phase consisted of methanol–water (97:3, v/v). It was buffered to pH 5.6 with acetic acid. The mobile phase was premixed, vacuum-filtered through a 0.45-µm nylon Millipore membrane (Millipore), and degassed by ultrasonication for 15 min before use. The flow rate was set at 2 mL/min. After equilibration with the solvent to obtain a stable baseline (20–30 min), aliquots of samples were injected. The total run time was 10 min, and injections were made 10 min apart to allow complete column re-equilibration. The absorbance of the eluent was monitored at 284 nm with a detection sensitivity of 0.01 aufs. Chromatography was performed at 25°C ± 0.5°C.

Preparation of the standard solutions

Drug concentrations in the working standard solutions chosen for the calibration curve were 15, 20, 25, 30, 60, 90, 120, 150, 180, and 210 µg/mL. Vitamin E acid succinate (appropriate accurately weighted amount for each concentration) was dissolved in 50 mL ethanol in a dark volumetric flask and stirred for 5 min, and 1.0 mL of this solution was diluted with 15 mL of ethanol to obtain the standard solutions. All samples were filtered through a 0.45-µm nylon filter. Previously, no adsorption of the drug on the filters was confirmed by measuring aliquots of the samples, with and without filtration. Dark tubes and dark volumetric flasks were tightly capped until analysis to protect the samples from light. The absorbances of solutions were measured at the typical maximum wavelength, 284 nm, using molar extinction coefficient in ethanol ($E_{1\%}$, 1 cm = 38.5) to spectrophotometrically confirm the accurate concentration of each working standard solution.

Preparation of the sample solutions

The vitamin E acid succinate content of the microspheres was quantitatively determined by an only one-step procedure. Recovery of vitamin E succinate from the microspheres before its quantitation was performed according to the method proposed by Benita et al. (22), applied to drugs soluble in organic solvents, but modified for vitamin E acid succinate. This procedure did not require repeated treatment, thereby saving time and minimizing sample decomposition and artifact formation.

Briefly, an accurately weighted amount of microspheres (10 mg) was vortex-mixed in dark tubes for 1 min with a small volume of methylene chloride (1 mL). Then, 15 mL of ethanol was added and mixed in a vortex mixer for 1 min to precipitate the polymer and dissolve vitamin E acid succinate. Different volumes of ethanol (9, 10, 12, 15, 17, and 19 mL) were tested to optimize the complete precipitation of the polymer. The mixture was centrifuged at 8500 × g for 25 min, and the supernatant was filtered

through a 0.45-µm syringe filter. The vitamin E acid succinate content in the filtrate was determined by HPLC. All of the samples were protected from light as previously described.

Method validation

A prospective validation protocol for analytical procedures was applied to this HPLC method as reported (23–28). The assay was validated with respect to linearity, range, limit of quantitation (LOQ) and LOD, accuracy, precision, recovery, specificity, and ruggedness. The stability of vitamin E acid succinate was also studied. Analysis of variance (ANOVA) was used to verify the validity of the method.

Linearity, range, LOD, and LOQ

The calibration curves were obtained from ten different concentrations of the standard solutions (15–210 µg/mL). The solutions were prepared in triplicate. Each concentration was injected twice. The linearity was evaluated by linear regression analysis, which was calculated by the least-square regression method. LOD and LOQ were determined on the basis of the response and slope of the regression equation.

Accuracy and precision

Accuracy was evaluated at ten different concentrations (15, 20, 25, 30, 60, 90, 120, 150, 180, and 210 µg/mL) on three different days. It was also determined by analyzing six replicates of sample solutions on three different days. Accuracy was defined as:

$$M \pm (SD/\sqrt{n}) \quad \text{Eq. 1}$$

where M was the mean potency value from recovery test, SD was the standard deviation, the Student's t was $t(0.05,29) = 2.045$, and n was the number of replicates.

The precision of the assay was determined by repeatability (intraday) and intermediate precision (interday) and was expressed as relative standard deviation percentage (RSD%) of a series of measurements. Repeatability was evaluated by assaying samples at the same concentration and during the same day. The intermediate precision was studied by comparing the assays on three different days. Six different sample solutions of three concentrations (15, 120, and 210 µg/mL) were prepared and assayed. The results were analyzed with the single-point calibration of the standard solution.

Standard addition and recovery

For recovery studies, a known concentration of analyte was added to 10 mg of nonloaded microspheres and treated as described in preparation of the sample solutions. Final concentrations of vitamin E acid succinate after the process resulted in 15, 120, and 210 µg/mL. Vitamin E acid succinate recovered from the microspheres was determined in triplicate for each concentration.

Specificity

Chromatograms of microspheres without vitamin E acid succinate and other compounds such as vitamin E were studied to verify that none of these products interfered with vitamin E acid succinate and that the degradation products of the drug did not interfere with its quantitation.

Ruggedness

The ruggedness of the method was evaluated by the analysis of several batches of microspheres under different experimental conditions such as changes in the composition of the mobile phase and its pH. The method was applied for a period of 17 months using several analytical columns (including a different column, Spherisorb ODS-2, 250- × 4.0-mm i.d., 5 µm), guard columns, and batches of chemical agents. The effect on the retention time and peak parameters were studied.

Stability

Vitamin E acid succinate must be protected from light (29), so it is important to minimize sample exposure to light during preparation and storage. The stability of vitamin E acid succinate was studied in ethanol at three different concentrations (15, 120, and 210 µg/mL) prepared in triplicate. Samples, in dark tubes, were stored at 25°C for 48 h, with observation for a change in the chromatograms compared with freshly prepared solutions.

Results and Discussion

The chromatographic conditions were adjusted to provide the best performance of the assay. Vitamin E acid succinate is practically insoluble in water. Therefore, the primary constituent of the mobile phase should be a weak organic solvent with low viscosity. These criteria limit the choices to methanol and acetonitrile. Methanol has been widely used for vitamin E quantitation because it yields higher recoveries than acetonitrile (19). To reduce the complexity of analytical conditions for continuous measurement, we used a methanol-based solvent as the mobile phase with water as a modifier. Several proportions of the mobile phase constituents (from 99.5:0.5 to 75:25 methanol–water), buffered at different pH values, were checked to establish the optimum separation and highest analytical sensitivity for vitamin E acid succinate. As it is known, by increasing the water content with respect to methanol, the retention time became longer. The mobile phase was buffered because of the strong carboxilate group in the chemical structure of vitamin E acid succinate (a weakly acidic phenolic OH such as vitamin E would not need to be buffered).

The best results were obtained with the conditions previously reported in the Experimental section. Vitamin E acid succinate eluted in a symmetrical peak and separated from the solvent front with a mean retention time of 6.5 min, which was stable in all of the analyzed samples. The pH of the mobile phase played a key role in the stability of the vitamin E acid succinate during the process. This was confirmed in our laboratory by assaying different mobile phases and buffering at different pH values, as previously commented. Consequently, the mobile phase was buffered at pH 5.6 ± 0.5 to avoid peak distortion phenomena in HPLC determination.

The present method avoids the use of gradient elution as well as

online solvent-mixing techniques. It is well known that the use of a gradient system combined with online mixing for vitamin determination often leads to poor reproducibility. Because of the sensitivity of vitamins to the modifiers, slight changes in the mobile phase composition would lead to a change in retention time. Furthermore, the differences in solvent refractive index cause an unstable chromatographic baseline. Online mixing of methanol and water also leads to out-gassing at the detector flow cell and increases baseline noise.

A guard column was also used to protect the analytical column and keep the retention time and peak resolution reproducible. In previous experiments carried out without the guard column, the retention time of vitamin E acid succinate seemed to gradually increase from injection to injection. This is probably attributable to the deposition of the compound on the column, causing a change in the surface characteristics of the stationary phase.

The quantitation of the vitamin was carried out by the external standard method using peak areas. We chose to work without an internal standard. The use of external standards was chosen, mainly, for two reasons: first, pro-oxidant impurities could be occasionally developed in the solvents, causing oxidative losses that cannot be monitored with an internal standard, and, second, because the molecules proposed as internal standards might not be entirely separate from the analytes of interest. A quantitative working standard containing 100 µg/mL of vitamin E acid succinate was injected frequently among samples to account for changes in detector sensitivity.

The calibration curves for vitamin E acid succinate were prepared by plotting concentration versus peak area. The calibration curve obtained from three independent experiments was $y = 4469.87x + 8130.17$. The standard error of intercept was 4680.26 and the intercept confidence interval –1455.01 – 17715.36. The standard error of slope was 41.48, and the slope confidence interval (95%) was 4384.90 – 4554.85. Linear relationships were obtained over the concentration range of 15–210 µg/mL, with a correlation coefficient (r) of 0.9988.

The lowest evaluated concentration demonstrating a precision and accuracy of less than 6% was regarded as the LOQ of the present assay. An LOQ of 15 µg/mL was observed for this assay. The experimental detection limit defined as the lowest concentration of analyte that can be distinguished from the noise level was 3 µg/mL (s/n = 2:1), indicating a high sensitivity of the method.

The validity of the assay was verified by means of ANOVA. Equality of variances (Cochran test) was not significant ($0.2928 < 0.4450$; $\alpha = 0.05$). There is linear regression ($11606.67 > 4.20$; $\alpha = 0.05$) and no significant lack of fit ($0.38 < 2.45$; $\alpha = 0.05$).

The accuracy of the method was determined, and the mean recovery was 100.23% ± 1.16% for the standard solutions (Table I), showing an agreement between the true value and the experimental value. The accuracy of the assayed sample solutions varied from 96.17% to 102.25%, with a mean value of 98.54% ± 2.59%.

Precision determined under the same conditions of work (intra-assay) and under different experimental conditions (interassay) was expressed by the coefficients of variation (CV_r for intra-assay and CV_R for interassay), being 2.08% and 2.32%, respectively. The experimental values obtained for the determination of vitamin E acid succinate are shown in Table II. The mean value of the obtained results was 99.31% ± 2.24% (97.00-103.48%), considering that the method was reliable. The standard addition and recovery study from the three assayed concentrations resulted in a mean percentage of recovered vitamin E acid succinate of 99.52% ± 0.81%, demonstrating high process efficiency.

Chromatograms of nonloaded microspheres showed no peaks in the region in which vitamin E acid succinate eluted, indicating specificity of the method against polymer interference. Furthermore, no interference of vitamin E acid succinate with other compounds such as vitamin E was observed ($R_S > 2$). This fact is extremely relevant for the evaluation of drugs included in microparticles.

The quality control of several batches of microspheres was used to evaluate the ruggedness of the method. During these assays, the retention time was not changed and the peak symmetry was conserved. When comparing the elution profile of a standard solution with a chromatogram of vitamin E acid succinate-loaded

Table I. Accuracy of the HPLC Assay for the Standard Solutions of Vitamin E Acid Succinate

Concentration added (µg/mL)	%Recovery (mean ± SD)
15	97.33 ± 2.18
20	104.33 ± 2.26
25	100.45 ± 3.64
30	101.01 ± 2.68
60	100.60 ± 1.33
90	100.03 ± 1.68
120	99.86 ± 3.95
150	96.36 ± 3.66
180	102.19 ± 2.21
210	100.16 ± 1.89

	Calculated values	Tabulated values	Significance
Equality of variances*	0.2159	0.4450	NS†
Means validation‡	2.07	2.39	NS

* Cochran test.
† No significance ($\alpha = 0.05$).
‡ F Snedecor test.

Table II. Results of the Precision Study

Concentration added (µg/mL)	%Recovery (mean ± SD)	Variance	CV (%)
15	100.71 ± 1.96	(4.28) Intra-assay	CV_r 2.08
120	99.14 ± 1.55	(1.04) Intergroup	
210	98.08 ± 2.56	(5.33) Interassay	CV_R 2.32
Mean recovery	99.31 ± 2.24%		

	Calculated value	Tabulated value	Significance
Equality of variances*	0.5129	0.7071	NS†

* Cochran test.
† No significance ($\alpha = 0.05$).

microspheres, there was no modification in the chromatogram profile (Figures 1 and 2). These facts suggest that the method did not change with time or conditions.

The data obtained from the stability studies showed that sample solutions at three different concentrations (15, 120, and 210 µg/mL) were stable for up to 48 h at 25°C with RSD values of 2.10%, 1.53%, and 1.18%, respectively, and no degradation took place when the samples were prepared.

The proposed method was applied to determine vitamin E acid succinate content in the studied PLGA microspheres batches (n = 20). The loading efficiency ranged from 93.83% to 97.60%, with a mean value of 96.53% ± 1.31% (87.75 µg vitamin E acid succinate/mg microspheres), which can be considered a high loading efficiency with a mean yield of microspheres production greater than 60%.

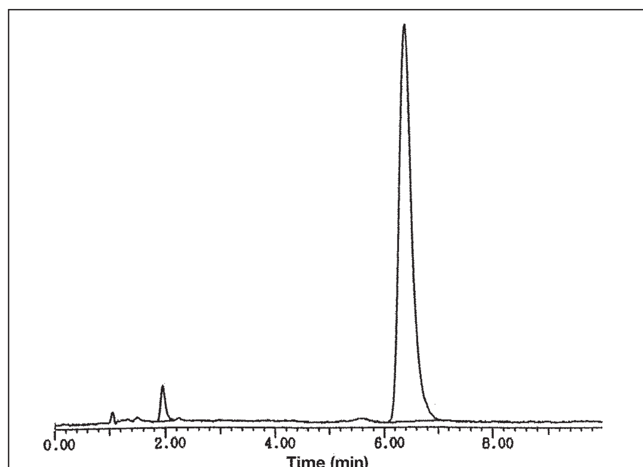

Figure 1. A typical HPLC chromatogram corresponding to a vitamin E acid succinate standard solution (120 µg/mL). Retention time, 6.48 min; chromatographic conditions, Extrasil ODS-2 analytical column (250- × 4.0-mm i.d., 5 µm) and ODS guard column; mobile phase, (pH 5.6) methanol–water (97:3 v/v); flow rate, 2 mL/min; and UV–vis detection at 284 nm.

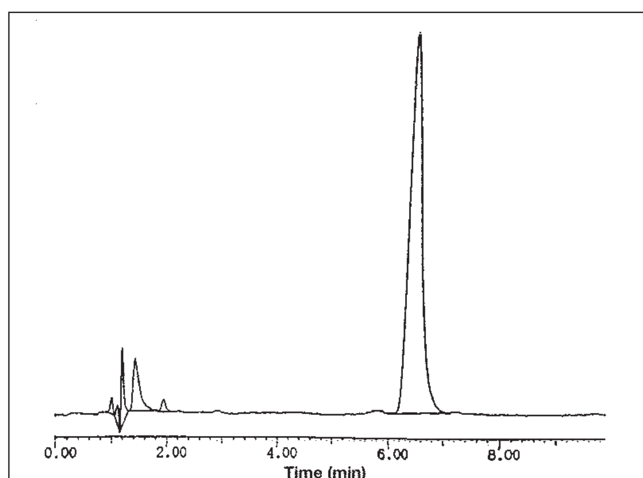

Figure 2. A typical HPLC chromatogram corresponding to a sample of vitamin E acid succinate-loaded microspheres. Retention time, 6.51 min; chromatographic conditions, Extrasil ODS-2 analytical column (250- × 4.0-mm i.d., 5 µm) and ODS guard column; mobile phase, (pH 5.6) methanol–water (97:3 v/v); flow rate, 2 mL/min; and UV–vis detection at 284 nm.

Conclusion

The present HPLC method can be considered simple, rapid, and easy to apply, and this makes it quite suitable for routine analysis of vitamin E acid succinate in biodegradable microspheres. It involves a single-step procedure for the preparation of the samples and direct injection. The sample preparation and analytical method are short (mean retention time, 6.5 min). Single-point calibration was chosen for analysis because of its simplicity and wide use in the pharmaceutical industry for uniformity analysis. This method allows the detection and quantitation of vitamin E acid succinate in microspheres but can also be used for the determination of the drug in other pharmaceutical dosage forms with reliability.

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