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# An HPLC method for quantitation of perillyl alcohol in a topical pharmaceutical cream formulation

Abhishek Gupta[a], Steven P. Stratton[b], Paul B. Myrdal[c,*]

[a] *Cardinal Health, Inhalation Formulation, P.O. Box 13341, Research Triangle Park, NC 27709, USA*
[b] *Arizona Cancer Center, University of Arizona, 1515 N Campbell Ave, Tucson, AZ 85724, USA*
[c] *College of Pharmacy, University of Arizona, 1703 E. Mabel St., Tucson, AZ 85721, USA*

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**Abstract**

A reverse phase high performance liquid chromatographic method for quantitation of perillyl alcohol in a topical cream pharmaceutical formulation was developed. Previously reported methods for analyzing drugs in lipid formulations are relatively complex and time consuming, with extraction, purification and derivatization involved. Through a simple dilution of the cream formulation in isopropyl alcohol, the present assay method enables the direct injection of the samples, on an Alltima C18 5 μ, 150 mm × 2.1 mm, narrow bore column (Alltech Associates, Deerfield, IL). The method includes an isocratic run with acetonitrile–water (40:60, v/v) at 0.35 mL/min for 12 min, followed by a gradient wash with isopropyl alcohol for 20 min, to ensure that all formulation excipients are eluted. Ultraviolet detection was performed at 210 nm with a retention time for perillyl alcohol of 7 min. The high sensitivity assay utilizes a small (5 μL) injection volume for the accurate and precise analysis of perillyl alcohol from a complex cream formulation.


*Keywords:* Perillyl alcohol; RP-HPLC method; Cream formulation; Topical formulation

## 1. Introduction

Perillyl alcohol or *p*-mentha-1,8-dien-7-ol or 4-isoprophenyl-cylcohexenecarbinol (POH) has been shown to be efficacious against the formation and progression of a variety of cancers. In animal studies it has been shown to be therapeutic for pancreatic, mammary and liver tumors and chemopreventive for colon, skin and lung cancer [1–5]. It has also been shown to inhibit photocarcinogenesis in a nonmelanoma model of mouse skin carcinogenesis and in a UVB-induced skin carcinogenesis model [6,7]. Preclinical trial results for POH demonstrated that monoterpenes have low toxicity and that topical application of POH is effective in skin cancer models [6].

In order to develop POH for topical use, it was necessary to formulate it in a topical formulation. Due to their biphasic nature and large number of excipients present, topical formulations are complex to analyze. The most common methods to analyze drug content in topical (lipoid) formulations include extraction of the drug from the formulation using an organic solvent (e.g., hexane, chloroform) followed by quantitation using normal phase chromatography [8–16]. These processes are time consuming and generally allow poor separation of the drug from the formulation excipients. The aim of this work was to develop a quantitative RP-HPLC method for the determination of the POH content in topical formulations.

## 2. Experimental

### 2.1. Materials

*R*-(+)-Perillyl alcohol and Glycerin, USP were obtained from Aldrich Chemical Company (Milwaukee, WI). White petrolatum, USP and mineral oil (light), NF, were obtained

* Corresponding author. Tel.: +1 520 626 3847; fax: +1 520 626 4063.
*E-mail address:* myrdal@pharmacy.arizona.edu (P.B. Myrdal).

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from Penreco (Dickinson, TX). Lanolin alcohol, NF and PPG-2-myristyl ether propionate, were obtained from Croda Inc. (Parsipanny, NJ). Stearic acid, USP and isopropyl palmitate were obtained from Uniqema (New Castle, DE). Paragon III was obtained from McIntyre Group Ltd. (University Park, IL). Distilled water, USP was obtained from Baxter Healthcare Corporation (Deerfield, IL). Triethanolamine, USP was obtained from Sigma Chemical Company (St. Louis, MO). Propyl paraben was obtained from Ruger Chemical Company (Irvington, NJ). High performance liquid chromatographic assay (HPLC) grade acetonitrile and isopropyl alcohol were obtained from Burdick and Jackson (Muskegon, MI). All the other chemicals were analytical or HPLC grade.

### 2.2. Formulation

The POH cream formulation is an oil-in-water emulsion and has been described elsewhere [17]. Briefly, the cream is a complex formulation consisting of white petrolatum, lanolin alcohol, PPG-2-Myristyl ether propionate, light mineral oil, triethanolamine, paragon III, glycerin, stearic acid, isopropyl palmitate and water.

### 2.3. Instrumentation and chromatographic conditions

The HPLC system consisted of a Waters 2690 separation module (Waters, Milford, MA) coupled with a Waters 996 Photodiode array (PDA) detector. An Alltima C18 5 μ, 150 mm × 2.1 mm, narrow bore column (Alltech Associates, Deerfield, IL) was used in this study. These columns can provide increased mass sensitivity and reduce consumption of solvents. In addition they have been reported to have stable bonded phases for longer column life and virtually no silanol activity, producing sharp and symmetrical peaks (Alltech Associates, Deerfield, IL). Initial isocratic conditions of acetonitrile–water (40:60, v/v) provide resolution of the drug from the internal standard (propyl paraben) and other formulation excipients. The drug had a retention time of 7.0 min whereas propyl paraben had a retention time near 5.3 min. The isocratic conditions were maintained for 10 min and followed by a linear gradient (2 min) with isopropyl alcohol until a mobile phase of 100% isopropyl alcohol was obtained. In order to elute very nonpolar formulation excipients from the column, the isocratic conditions with 100% isopropyl alcohol were maintained for 18 min and then the composition of mobile phase was returned to the starting conditions. The column was allowed to equilibrate with acetonitrile–water (40:60, v/v) before the next sample was injected.

The instrumentation details and gradient HPLC assay method used to analyze POH are as follows:

| | | |
|---|---|---|
| Column | Altima C18 5 μ | |
| Length | 150 mm × 2.1 mm | |
| Flow rate | 0.35 mL/min | |
| Loop volume | 5.0 μL | |
| Wavelength | 210 nm | |
| Retention time | 7.0 min | |
| Total run time | 45 min | |
| Temperature | 30 ± 2 °C | |
| Mobile phase | ACN–water (40:60, v/v) | 0–10 min |
| | ACN–water (40:60, v/v) to IPA (100, v/v) | 10–12 min |
| | IPA (100, v/v) (0.15 mL/min) | 12–30 min |
| | ACN–water (40:60, v/v) (0.1 mL/min) | 30–33 min |
| | ACN–water (40:60, v/v) (0.35 mL/min) | 33–45 min |

### 2.4. System suitability

The analytical column was equilibrated with the initial mobile phase composition and six injections were made with a standard solution, under the conditions described above. In order to evaluate the system suitability six consecutive injections were made with the same sample, with the same equipment and on the same day.

### 2.5. Preparation of standard solutions-calibration curve

Stock solutions of POH and propyl paraben were prepared in IPA and stored at 4 °C until use. The concentration of the POH stock solution was 404 μg/mL and it was diluted with IPA to obtain a concentration range of 12.75–204 μg/mL, after combining equal volumes of POH solution and internal standard solution. These standards were stable for more than 2 weeks when stored at 4 °C. Three standard curves were prepared daily for this concentration range, in order to evaluate the linearity. The peak area ratio of POH to internal standard (propyl paraben) was plotted against POH concentration to construct the standard curve.

### 2.6. Sample preparation

Three different cream formulations were manufactured having different concentrations of POH (0.304, 0.761 and 1.522% w/w) using propyl paraben (0.126% w/w) as the internal standard for each. Formulations were sampled by taking 0.250 mg of each of these formulations and diluting with 20 mL of isopropyl alcohol. Each of these samples were then sonicated for 20 min (Bransonic-1510R, Bransonic Ultrasonics Corp., Danbury, CT). The samples were inspected with a laser tyndall beam in order to make sure that all the components in the vials were in solution. Five microlitres of aliquots of these formulation samples were injected onto the HPLC analytical column for analysis.

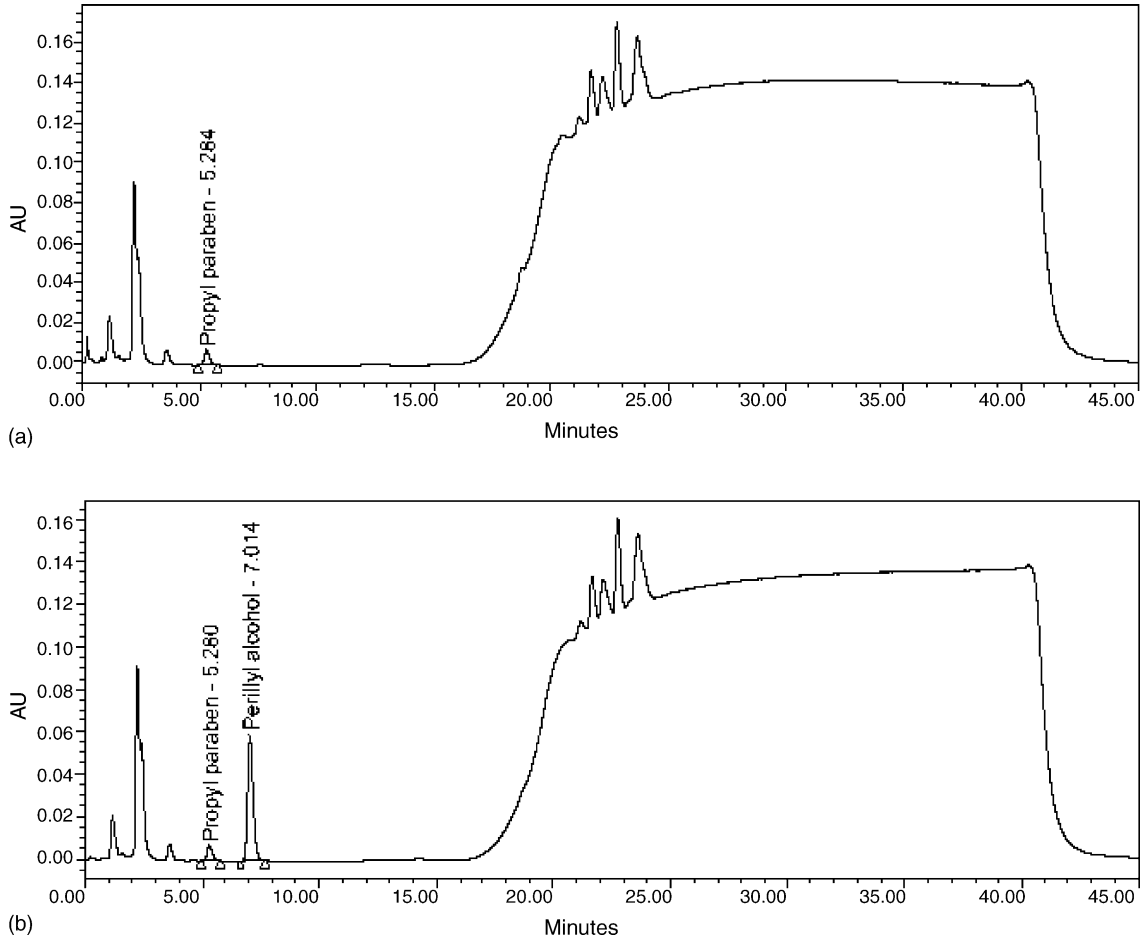


Fig. 1. Example chromatograms for the cream formulation, not containing POH (a) and containing POH (b).

## 3. Results

### 3.1. Performance characteristics of the analytical method

The analytical peaks of POH and propyl paraben were well resolved from each other. Fig. 1 shows the chromatograms for the blank cream formulation (placebo) and for the cream formulation having POH. The optimized chromatographic conditions resulted in a retention time of 7.0 min for POH and 5.3 min for propyl paraben. The ICH and FDA [18,19] guidelines were taken into consideration while evaluating the analytical method. In order to demonstrate the satisfactory nature of the method, the following protocol was implemented during the development and evaluation.

### 3.2. System suitability

To determine system suitability, six consecutive injections were made with a standard solution, before each sample set was analyzed. This testing was conducted to ensure optimum system function parameters on that particular day. Quantitation was performed using peak area% of the drug peak relative to the internal standard peak. The coefficient of variation (CV) was used as a measure of precision. The tailing factors for all the measured peaks were between −0.85 and +1.20. All the peaks were well resolved with a resolution of 1.5 or greater.

Table 1 lists the system suitability data for six injections on five different days, along with their standard deviations (S.D.) and coefficient of variation. The S.D. and CV, over a period of 5 days were 0.00045 and 0.69, respectively.

Table 1
Intra-day and inter-day system suitability data: PA/PP area% represents the ratio of the peak area% of the POH peak to the propyl paraben (internal standard) peak

| Day | *N* | PA/PP (area%) | S.D. | CV (%) | *p*-Value |
|---|---|---|---|---|---|
| 1 | 6 | 0.06 | 0.00051 | 0.79 | 0.00042 |
| 2 | 6 | 0.06 | 0.00061 | 0.95 | 0.00050 |
| 3 | 6 | 0.06 | 0.00024 | 0.37 | 0.00019 |
| 4 | 6 | 0.06 | 0.00024 | 0.37 | 0.00019 |
| 5 | 6 | 0.06 | 0.00017 | 0.26 | 0.00014 |
| Inter-day | | | | | |
| Mean | 0.06 | | | | |
| S.D. | 0.00045 | | | | |
| CV (%) | 0.69 | | | | |

### 3.3. Sensitivity and limit of detection

The limit of detection and the limit of quantitation (LOD and LOQ, respectively) of the method were determined from the standard deviation of the response, of known concentrations of POH. The LOD is defined as the lowest drug concentration, which can be determined and calculated as three times the variation in the measured response [19]. For this method the LOD was calculated to be 2.25 μg/mL. In the same way, LOQ was estimated as ten times the variation in the measured response [19] and was calculated to be 6.88 μg/mL. Experimentally the LOQ was determined to be 3.75 μg/mL.

### 3.4. Selectivity and specificity

The selectivity of the developed RP-HPLC method for the determination of POH in pharmaceutical formulations was investigated at the retention times of the analyte and the internal standard. It is evident from the placebo (see Fig. 1) that the excipients in the formulation do not cause any interference with the POH peak, and both POH and internal standard were well resolved. Also in order to confirm the selectivity of the method for POH, forced degradation studies were conducted in aqueous buffer systems and organic solvents. None of the potential degradation products were found to interfere with the POH peak under the initial isocratic mobile phase solvent conditions [17]. Under these conditions, POH was observed to be well resolved from other formulation ingredients and any potential degradation products.

The specificity of the method for POH was confirmed by library spectra matching and mass spectrometry. A library spectra match was performed on each of the POH peaks using a Waters 996 photo diode array detector, in order to check for peak purity. The peak purity data confirmed the absence of any impurities coeluting with POH. In addition aliquots of the POH formulation samples were collected corresponding to the elution time of the POH peak. The eluates were then combined and analyzed using Varian Saturn 2000 GC–MS (electron ionization) in the positive ion mode (Varian Inc., Palo Alto, CA). The analysis confirmed the identity and uniqueness of the POH peak.

### 3.5. Linearity

The calibration curve for POH was prepared in the concentration range of 12–200 μg/mL. The calibration curve demonstrated the satisfactory and consistent behavior of the method. The data for this concentration range was analyzed using least-squares regression analysis, and the results are shown in Table 2 Table 2(a) and (b). Linearity was determined by plotting a standard curve using the ratio of POH peak area to propyl paraben (internal standard) peak area, versus the corresponding drug concentration in the sample. All the calibration curves were linear on five different days, with a correlation coefficient $r \geq 0.9999$ and with confidence intervals less than $p = 0.05$. The intercepts were not significantly different from zero, therefore, the least-squares regression line was used without an intercept. In addition, relative error in each concentration was calculated from the calibration curve and ranged from 0.17 to 1.35 (Table 2(b)). The relative error provides a measure of the difference between the experimental and calculated values and thus a measure of the scatter of the data about the best fit-line.

### 3.6. Accuracy

Accuracy of the analytical assay was determined by directly analyzing the formulations with the active ingredients

Table 2
Linearity data

| Day | *N* | Slope | S.D. | CV (%) | 95% C.I. | *r* |
|---|---|---|---|---|---|---|
| Part (a) | | | | | | |
| 1 | 3 | 0.00125 | 2.88E−05 | 0.0057 | 3.25E−05 | 0.9999 |
| 2 | 3 | 0.00125 | 2.05E−11 | 1.65E−06 | 2.32E−11 | 0.9999 |
| 3 | 3 | 0.00125 | 2.06E−11 | 1.65E−06 | 2.32E−11 | 0.9999 |
| 4 | 3 | 0.00125 | 2.06E−11 | 1.65E−06 | 2.32E−11 | 0.9999 |
| 5 | 3 | 0.00126 | 2.06E−11 | 1.65E−06 | 2.32E−11 | 0.9999 |
| Inter-day | | | | | | |
| Mean | 0.001252 | | | | | |
| S.D. | 4.472E−06 | | | | | |
| CV (%) | 0.3571994 | | | | | |

| Sample (*N* = 15) | Concentration added (μg/mL) | Concentration recovered (μg/mL)[a] | S.D. | CV (%) | R.E. |
|---|---|---|---|---|---|
| Part (b) | | | | | |
| 1 | 12.75 | 12.59 | 0.22 | 1.73 | 1.35 |
| 2 | 25.5 | 25.21 | 0.29 | 1.15 | 1.17 |
| 3 | 51 | 50.40 | 0.94 | 1.85 | 1.17 |
| 4 | 102 | 102.11 | 0.90 | 0.88 | 0.65 |
| 5 | 204 | 204.35 | 0.79 | 0.38 | 0.17 |

[a] Calculated using slopes from Table 2a.

Table 3
Accuracy data

| Day | *N* | Concentration recovered (%, w/w) | S.D. | CV (%) | *p*-Value | R.E. |
|---|---|---|---|---|---|---|
| (1) 0.304% (w/w) | | | | | | |
| 1 | 6 | 0.306 | 0.001 | 0.39 | 0.095 | 0.54 |
| 2 | 6 | 0.305 | 0.004 | 1.32 | 0.218 | 0.34 |
| 3 | 6 | 0.303 | 0.001 | 0.39 | 0.064 | −0.41 |
| 4 | 6 | 0.305 | 0.004 | 1.34 | 0.220 | 0.33 |
| 5 | 6 | 0.303 | 0.003 | 1.09 | 0.179 | −0.23 |
| Inter-day | | | | | | |
| Mean | 0.304 | | | | | |
| S.D. | 0.001 | | | | | |
| CV (%) | 0.41 | | | | | |
| R.E. | 0.12 | | | | | |
| (2) 0.761% (w/w) | | | | | | |
| 1 | 6 | 0.781 | 0.001 | 0.14 | 0.059 | 2.67 |
| 2 | 6 | 0.784 | 0.004 | 0.48 | 0.201 | 3.03 |
| 3 | 6 | 0.786 | 0.002 | 0.31 | 0.130 | 3.25 |
| 4 | 6 | 0.786 | 0.001 | 0.18 | 0.076 | 3.25 |
| 5 | 6 | 0.785 | 0.004 | 0.49 | 0.972 | 3.11 |
| Inter-day | | | | | | |
| Mean | 0.784 | | | | | |
| S.D. | 0.002 | | | | | |
| CV (%) | 0.23 | | | | | |
| R.E. | 3.06 | | | | | |
| (3) 1.522% (w/w) | | | | | | |
| 1 | 6 | 1.513 | 0.008 | 0.53 | 0.44 | −0.56 |
| 2 | 6 | 1.548 | 0.014 | 0.91 | 0.76 | 1.67 |
| 3 | 6 | 1.562 | 0.007 | 0.42 | 0.35 | 2.64 |
| 4 | 6 | 1.505 | 0.014 | 0.93 | 0.76 | −1.07 |
| 5 | 6 | 1.526 | 0.018 | 1.15 | 0.97 | 0.24 |
| Inter-day | | | | | | |
| Mean | 1.531 | | | | | |
| S.D. | 0.024 | | | | | |
| CV (%) | 1.54 | | | | | |
| R.E. | 0.59 | | | | | |

and by adding known amount of drug by weight to the placebo formulation. The results were identical and further evaluation was performed by analyzing the percentage of the theoretical drug recovered for the 0.304, 0.761 and 1.522% (w/w) POH formulations. The intra-day and inter-day accuracy along with the CV (%) and R.E. are summarized in Table 3. Deviation of the obtained result for the POH formulations, from the theoretical concentrations of 0.304, 0.761 and 1.522% (w/w) were within ±3.3%, during intra-day and inter-day analysis. The *p*-values (95% confidence interval) show that the experimental mean was not significantly different from the true value, during intra-day and inter-day analysis.

### *3.7. Precision*

To calculate the precision of the method, intra-day and inter-day tests were performed. The precision was measured in terms of the ratio of area% of POH peak to internal standard peak and was expressed as coefficient of variation. Intra-day and inter-day variability in the assay was determined by measuring 6 samples with three different concentrations, for 5 different days. The *p*-values (95% confidence interval) indicate that the results were not significantly different during intra-day and inter-day analysis. The inter-day CV values were 0.83 for 0.304% (w/w) formulation, 1.20 for 0.761% (w/w) formulation and 0.86 for 1.522% (w/w) formulation. The values along with S.D. and CV (%) are summarized in Table 4. From the results it is clear that the method is reproducible within the same day and between different days.

## 4. Discussion

A number of assays have been developed for the analysis of POH in plasma [17–22], however there are no published RP-HPLC methods for the quantitation of POH in pharmaceutical formulations. The method described in this report is the first analytical HPLC procedure that is suitable for quantitating POH in pharmaceutical formulations that are lipoid in nature. Most analysis methods for drug content determination in lipid formulations are complicated and require elaborate sample pre-treatment along with extraction and derivatization

Table 4
Precision data: PA/PP area% represents the ratio of the peak area% of the POH peak to the propyl paraben (internal standard) peak

| Day | *N* | PA/PP (area%) | S.D. | CV (%) | *p*-Value |
|---|---|---|---|---|---|
| (1) 0.304% (w/w) | | | | | |
| 1 | 6 | 3.74 | 0.06 | 1.52 | 0.09 |
| 2 | 6 | 3.68 | 0.06 | 1.55 | 0.09 |
| 3 | 6 | 3.75 | 0.04 | 1.17 | 0.07 |
| 4 | 6 | 3.73 | 0.07 | 1.77 | 0.11 |
| 5 | 6 | 3.70 | 0.07 | 1.89 | 0.12 |
| Inter-day | | | | | |
| Mean | 3.72 | | | | |
| S.D. | 0.03 | | | | |
| CV (%) | 0.83 | | | | |
| (2) 0.761% (w/w) | | | | | |
| 1 | 6 | 9.42 | 0.19 | 1.99 | 0.32 |
| 2 | 6 | 9.49 | 0.06 | 0.60 | 0.09 |
| 3 | 6 | 9.45 | 0.15 | 1.59 | 0.25 |
| 4 | 6 | 9.68 | 0.08 | 0.85 | 0.14 |
| 5 | 6 | 9.39 | 0.07 | 0.72 | 0.11 |
| Inter-day | | | | | |
| Mean | 9.49 | | | | |
| S.D. | 0.11 | | | | |
| CV (%) | 1.20 | | | | |
| (3) 1.522% (w/w) | | | | | |
| 1 | 6 | 19.27 | 0.22 | 1.14 | 0.37 |
| 2 | 6 | 19.00 | 0.13 | 0.66 | 0.21 |
| 3 | 6 | 18.95 | 0.25 | 1.33 | 0.42 |
| 4 | 6 | 19.28 | 0.05 | 0.23 | 0.08 |
| 5 | 6 | 18.97 | 0.11 | 0.56 | 0.18 |
| Inter-day | | | | | |
| Mean | 19.09 | | | | |
| S.D. | 0.16 | | | | |
| CV (%) | 0.86 | | | | |

[8–16]. This method does not require preliminary extraction or the expensive and potentially hazardous radiolabel-based assays [23,24]. The method utilizes a direct injection of the sample, thereby eliminating elaborate sample preparation prior to injection. The high sensitivity of the assay requires only a very small (5 μL) injection volume for the accurate and precise analysis of POH. The sample concentration can be determined accurately and precisely over a relatively broad concentration range using a small sample size (5 μL). Although the length of the method is 45 min, the retention time of the active is only about 7 min and the additional time is required for separation of the formulation ingredients. Isopropyl alcohol was used to elute the non-polar formulation ingredients and this isocratic run was conducted for about 20 min, taking a conservative approach. The length of the method can be reduced by about 10 min by reducing the time of the isocratic run with isopropyl alcohol. The utility of this assay can be extended to other lipoid formulations of POH, without making any substantial changes in the assay method. It is readily adaptable for assaying other similar formulations of POH and is versatile in its nature. In summary, the HPLC analysis method of POH described in the present study is characterized by sufficient accuracy, precision and reproducibility, as well as sensitivity and selectivity. The simplicity of the technique, the minimal volume requirement and the high sensitivity make this technique particularly attractive for the quantification of POH in pharmaceuticals.

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