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Stereo-specific analysis of a novel protein kinase C inhibitor

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Abstract: SPC-100270 drug substance ((2S,3S)-2-aminooctadecane-1,3-diol) is a synthetic sphingosine analogue possessing limited ultraviolet absorbance (UV) and two chiral centres. Analytical methodology employing derivatization of SPC-100270 with *o*-phthaldialdehyde (OPA) and a chiral thiol, N-acetyl-D-penicillamine, was developed to assess the isomeric purity of SPC-100270 in the presence of two of its three stereoisomers. Separation of the isoindole derivatives was achieved with isocratic reversed-phase column chromatography with detection at 330 nm. Under the conditions studied, the derivatization was complete within 3 h at 50°C. The standard and derivatized solutions were stable for 7 days under refrigerated conditions. The limit of detection was 0.036 μg SPC-100270 per ml and the limit of quantitation was 0.237 μg SPC-100270 per ml. The assay response was linear over a concentration range of 4.7–376 μg SPC-100270 per ml with a coefficient of determination of greater than 0.9999. The precision, ruggedness and specificity of the assay were acceptable for determination of SPC-100270 in the presence of its stereoisomers and under forced degradation conditions. The method has been applied successfully in two independent laboratories for quality control release and stability assessment of SPC-100270 drug substance for early clinical studies.

Keywords: *Sphingosine, isoindole, o-phthaldialdehyde, N-acetyl-D-penicillamine, chiral derivatization, reversed-phase chromatography.*

Introduction

SPC-100270 drug substance ((2S,3S)-2-amino-octadecane-1,3-diol), a synthetic analogue of the naturally occurring protein kinase C (PKC) inhibitor sphingosine, has been found to inhibit this enzyme at micromolar levels. SPC-100270 has shown both antiproliferative and anti-inflammatory activity in animal models and was developed for atopic dermatitis and psoriasis [1–3]. Additionally, SPC-100270 has shown promise as a chemopotentiating agent [4, 5].

SPC-100270 has a long, hydrophobic aliphatic chain, and a polar end consisting of vicinal hydroxyls and a weakly basic primary amino group. SPC-100270 possesses two asymmetric centres and, therefore, may exist as four stereoisomers. The chemical structures of SPC-100270 and its isomers are shown in Fig. 1. Although the racemic as well as stereochemically pure isomers display similar biological activity, the (2S,3S)-2-aminooctadecane-1,3-diol isomer was selected as the candidate for further development due to its favoured metabolic pathway (personal communication from Sphinx Pharmaceuticals). Early development efforts, therefore, required development of a stero-specific assay to determine the stereochemical purity of SPC-100270.

The synthetic scheme for SPC-100270 minimizes formation of SPC-102860 (2S,3R enantiomeric isomer) and production of SPC-103980 (2R,3S isomer) is unlikely due to functional group blocking. Therefore, achieving good separation of SPC-100580 (2R,3R isomer), SPC-102860 and SPC-100270 allows accurate determination of chiral purity.

Due to the limited absorbance response (UV) of SPC-100270, a chiral derivatization procedure was developed utilizing *o*-phthaldialdehyde (OPA) [6–10] and a chiral thiol, N-acetyl-D-penicillamine [11, 12], to covalently modify the primary amine of SPC-100270 to form the isoindole derivative (see Fig. 2). This derivatization introduces a chromophore/fluorophore for detection and a handle for chromatographic isomeric resolution by for-

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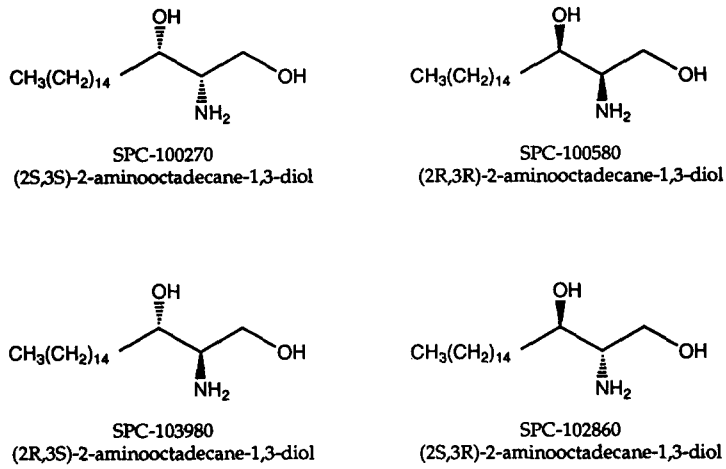

Figure 1
Stereochemical structure of SPC-100270 and its isomers.

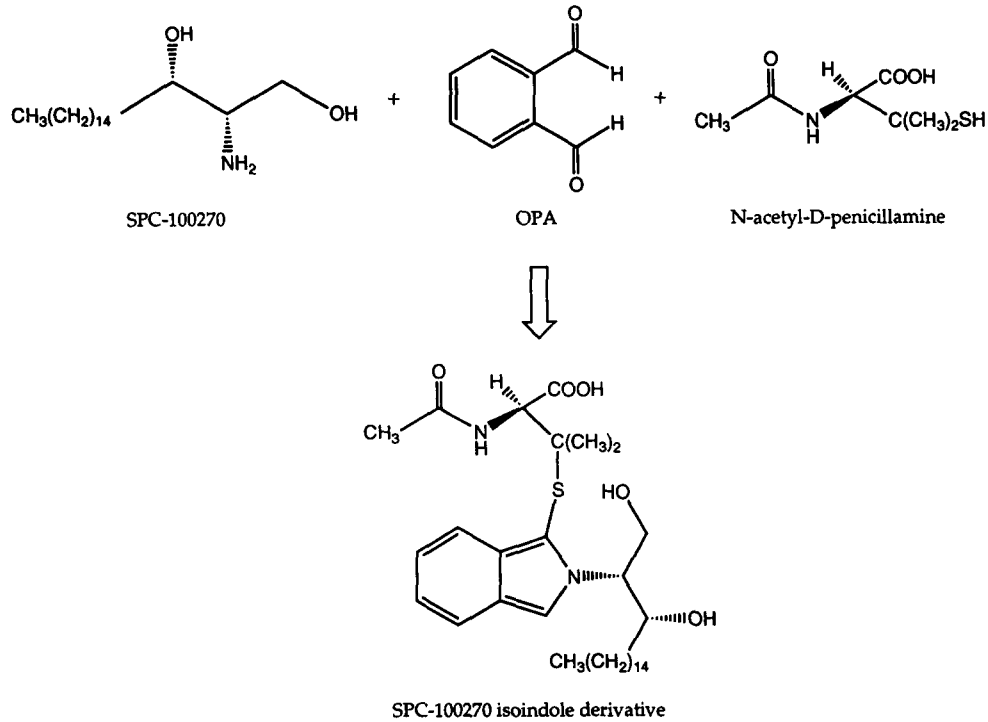

Figure 2
Chemical structures of SPC-100270, *o*-phthaldialdehyde (OPA), N-acetyl-D-penicillamine and the SPC-100270 isoindole derivative.

mation of diastereomers of the two possible enantiomeric pairs. Since UV detection of the isoindole derivatives provides adequate assay sensitivity and UV detection is ubiquitous in quality control laboratories, the assay was validated with UV detection. In this paper the development and validation of this stereospecific assay for use in early clinical development is presented.

Materials and Methods

Materials

SPC-100270 ((2S,3S)-2-aminooctadecane-1,3-diol), SPC-100580 ((2R,3R)-2-aminooctadecane-1,3-diol) and SPC-102860 ((2S,3R)-2-aminooctadecane-1,3-diol) were synthesized in Sphinx Pharmaceuticals laboratories. D,L-erythro-dihydrosphingosine (~99%)(racemic

mixture of SPC-102860 and SPC-103980) and *o*-phthaldialdehyde (OPA) (~99%) were obtained from The Sigma Chemical Company (St Louis, USA). N-acetyl-D-penicillamine (>99%) and N-acetyl-D,L-penicillamine (>98%) were obtained from Fluka Chemie AG (Switzerland). All other reagents were obtained from conventional sources.

Chromatographic system

A modular system consisting of a Shimadzu SCL-10A controller, SIL-10A autoinjector and sample cooler, SPD-10AV UV or SPD-M6A diode array detector, LC-10AS pump, and C-R4AX data processor was used with a Zorbax C8 LC column (4.6 × 250 mm, 5 μm particle size) and a Brownlee RP-8 guard cartridge for chromatographic analysis. The filtered and degassed eluent consisted of methanol–aqueous sodium acetate (pH 5.90; 50 mM) (80:20, v/v) at a flow rate of 1.5 ml min^{-1}. Detection was at 330 nm with ambient column and sample temperatures.

Methods

General derivatization and analysis procedure. One millilitre of a sample or standard solution of SPC-100270 in methanol was pipetted into a glass vial. Eight hundred microlitres of 200 mM aqueous sodium tetraborate buffer (pH 10.00), 40 μl of 30 mg ml^{-1} OPA in anhydrous ethanol, and 40 μl of 30 mg ml^{-1} N-acetyl-D-penicillamine in methanol were added to this solution. After mixing, the solution was held at 50°C for 3 h to complete the analyte derivatization. System suitability requirements performed before each experiment included a resolution of ≥3.0 for SPC-100270 and SPC-100580 and a tailing factor of ≤2.0 and theoretical plates ≥3500 for the SPC-100270 peak. All calculations were performed according to the current *United States Pharmacopeia.*

Determination of derivatization time. Twenty-five millilitres of a 350 μg ml^{-1} solution of SPC-100270 in methanol and 500 μl each of 350 μg ml^{-1} SPC-100580 in methanol and 700 μg ml^{-1} D,L-erythro-dihydrosphingosine in methanol were derivatized in a manner analogous to the general derivatization and analysis procedure described. The theoretical final concentration of each isomer of SPC-100270 relative to SPC-100270 is 2% by weight assuming a purity of 100% for the isomers. The vials were assayed over approximately 9 h at 30 min intervals to follow the derivatization of SPC-100270 and its isomers. The results of this study are presented in Fig. 3.

Standard solution stability. On the initial day of this experiment, 100 ml of 100 μg ml^{-1} SPC-100270 was prepared in methanol. Approximately 22 ml of this solution was derivatized in a manner analogous to the general derivatization and analysis procedure described to yield 50 ml of derivatized solution. Both the derivatized and underivatized solutions were stored at 2–8°C and analysed over a 7-d period to assess solution stability.

Limit of detection/limit of quantitation. The limit of detection was defined as the lowest SPC-100270 concentration with a signal-to-noise ratio of >3 from single injections of the derivatized analyte. The limit of quantitation was defined as the lowest SPC-100270 concentration at which the percentage relative standard deviation (RSD) for six injections of derivatized analyte was ≤2 and the signal-to-noise ratio was ≥10. Dilutions of a standard SPC-100270 solution were derivatized and analysed according to the general derivatization and analysis procedure described.

Linearity. SPC-100270 solutions ranging in concentration from 4.7 to 376 μg ml^{-1} were prepared in methanol. Samples of these solutions were derivatized and analysed in duplicate according to the general derivatization and analysis procedure described.

Precision/ruggedness. Six 350 μg ml^{-1} SPC-100270 samples were derivatized and analysed in duplicate according to the general derivatization and analysis procedure described to assess method precision. Method ruggedness was evaluated by having a second analyst derivatize and analyse identical samples on a different LC system. The means, standard deviations and %RSD for SPC-100270 and its isomers may be found in Table 1.

Specificity. Assay specificity was determined by derivatization of available synthetic precursors, process intermediates and isomers according to the general derivatization and analysis procedure described. These samples were assayed in the presence and absence of derivatized SPC-100270 to observe possible

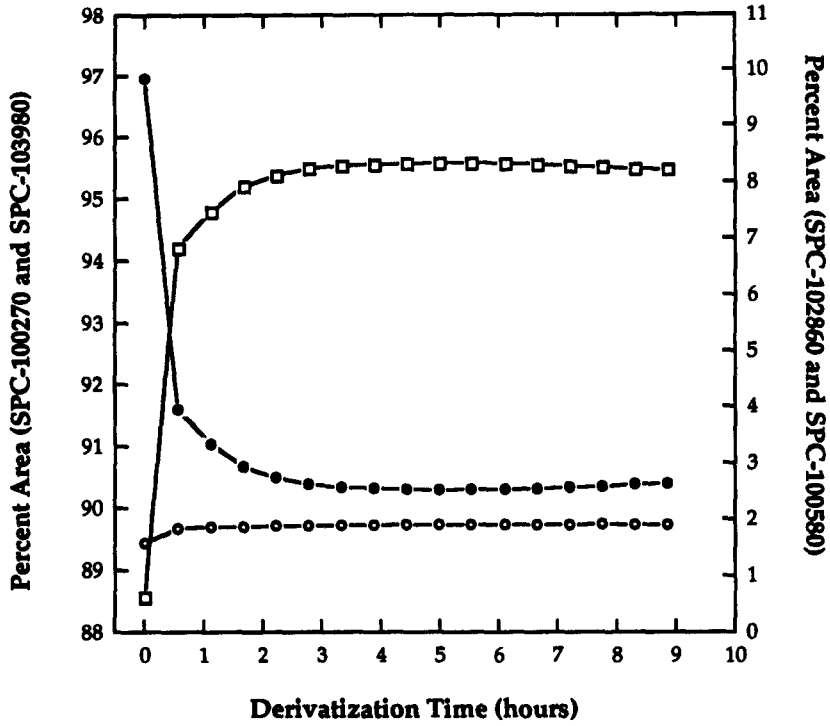

Figure 3
Dependence of the isoindole peak area of SPC-100270 and its isomers as a function of derivatization time; SPC-100270 and SPC-103980 (□), SPC-10058 (○), SPC-102860 (●).

Table 1
Precision data of percent total peak area for the isoindole derivatives of SPC-100270 and its isomers from two independent analyses (n = 6)

		SPC-100270 & SPC-103980	SPC-100580*	SPC-102860*
Mean (%)	Analyst 1	98.53	0.26	1.21
	Analyst 2	98.24	0.35	1.41
Standard deviation (%)	Analyst 1	0.01	0.005	0.01
	Analyst 2	0.07	0.04	0.04
Percentage RSD (%)	Analyst 1	0.01	1.92	0.82
	Analyst 2	0.07	11.43	2.84

* Mean relative to SPC-100270.

peak interference. SPC-100270 solutions were also subjected to thermal stress (60°C/24 h), acid and base (0.2 M HCl or 0.2 NaOH/60°C/ 24 h) and oxidation (3% H_2O_2/ambient temperature/15 min). The acid and base stressed solutions were neutralized prior to derivatization and assay. Peak purity was assessed with diode array detection.

Results and Discussion

The derivatization of SPC-100270 with OPA and N-acetyl-D-penicillamine introduces a handle for UV detection of the analyte while providing for chromatographic isomeric resolution by formation of diastereomers of the two possible enantiomeric pairs. The method as developed resolves SPC-100270 from two of the possible three diastereomers formed.

The synthetic scheme for SPC-100270 uses L-serine as the starting material and the stereochemistry at carbon atom 2 is maintained during synthesis. The final product is obtained by reducing the ketone formed at carbon atom 3 with sodium borohydride. Proper cooling during this reduction allows minimal formation of SPC-102860 ((2S,3R)-2-aminooctadecane-1,3-diol). Due to the presence of bulky protecting groups at the amine, formation of SPC-

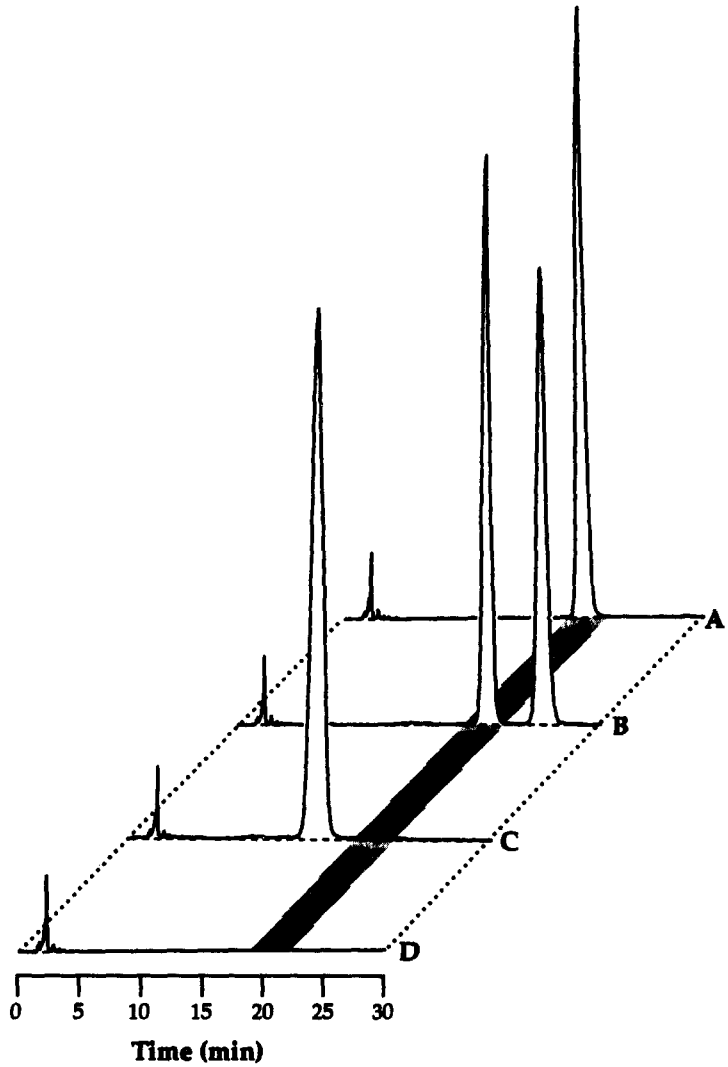

Figure 4
LC tracings of the isoindole derivatives of SPC-100270 and its derivatives; (A) SPC-100270, (B) SPC-103980 (left) and SPC-102860 (right), (C) SPC-100580, (D) methanol blank.

103980 ((2R,3S)-2-aminooctadecane-1,3-diol) is not likely. Therefore, the lack of separation of SPC-100270 from SPC-103980 should not be important in the quantitation of SPC-100270.

As can be seen in Fig. 4 and Table 2, SPC-100270 is well resolved from SPC-102860 and SPC-100580. SPC-103980, however, elutes with a retention time similar to SPC-100270. Since isomers of SPC-100270 were not analytically characterized as reference standards, their use in the LC analysis was limited to determination of relative retention volume. Due to the unknown purity of these isomers, the isomeric purity of SPC-100270 was determined on peak area basis relative to the resolved isomers. The use of percentage area for determination of isomeric purity is supported by the common isoindole chromophore. Diode array spectrophotometric analysis of derivatized SPC-100270 and its resolved isomers demonstrated that the UV absorbance spectra of these derivatives are identical (see Fig. 5).

Table 2
Retention times and diode-array spectrophotometric similarity indices for SPC-100270 and its isomers

	Retention time (min)	Similarity index
SPC-100270	19.73	1.000
SPC-100580	15.28	0.9998
SPC-103980	20.25	0.9997
SPC-102860	24.82	0.9998

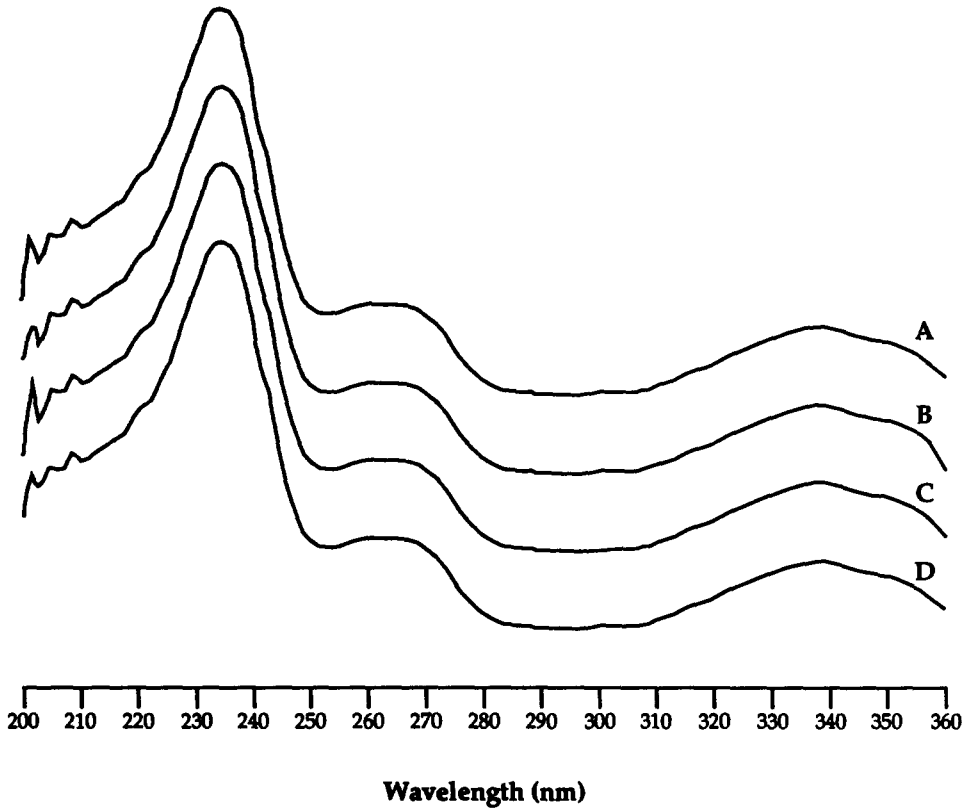

Figure 5
Diode array UV spectra of the HPLC peaks of SPC-100270 and its isomers; (A) SPC-100270, (B) SPC-100580, (C) SPC-102860, (D) SPC-103980.

The isomeric purity of the N-acetyl-D-penicillamine as obtained was reported to be >99%. Derivatization of SPC-100270 and its isomers with N-acetyl-D,L-penicillamine yields identical chromatography to that produced with N-acetyl-D-penicillamine. Therefore, the presence of any N-acetyl-L-penicillamine impurity in N-acetyl-D-penicillamine is not believed to affect the quantitation of SPC-100270.

The minimum derivatization time for the method was set at 3 h at 50°C. As can be seen in Fig. 3, the percentage area of SPC-100270 and its isomers plateau by approximately 3 h and remain relatively stable over the next 6 h. The stability of the isoindole derivative formed is surprising but not inconsistent with data generated employing other highly substituted thiols [13, 14]. The SPC-100270 and derivatized SPC-100270 standard solutions were stable over a 7-day period at refrigerated temperatures.

The limit of detection for SPC-100270 was 0.036 $\mu g\ ml^{-1}$ (signal-to-noise of 3.5) as measured by the height of the SPC-100270 peak and average baseline noise from a strip chart recorder. The limit of quantitation was determined to be 0.237 $\mu g\ ml^{-1}$ based on an RSD deviation of 1.61% and a signal-to-noise ratio of 18. Based on the common chromophore and relative peak shapes, the limit of quantitation for the associated isomers should be in the same order of magnitude.

The assay response for the SPC-100270 peak was found to have a linear dependence on SPC-100270 concentration over a range of 4.7–376 $\mu g\ ml^{-1}$. The regression line had an insignificant y-axis intercept (-2.51×10^3 area units, standard error = 1.54×10^4 area units, P-value = 0.873), a slope of 2.90×10^4 area units $\mu g^{-1}\ ml^{-1}$ and a coefficient of determination of 0.9999.

The method precision for six independently derivatized SPC-100270 samples analysed in duplicate demonstrates acceptable standard deviation for quantitation of SPC-100270 and its isomers. The ruggedness of the assay as determined by analysis of the same samples by a second analyst on a different LC system demonstrates the ability of the method to produce comparable results by independent analyses (see Table 1).

The three isomers of SPC-100270 along with a process intermediate and precursor to SPC-100270 were investigated for possible elution interference with SPC-100270. For samples in which SPC-100270 was present, the peak purity as determined by diode array analysis was noted. Peak purity calculations were based on comparison of the absorbance spectrum at the beginning, middle and end of the chromatographic peak. A peak purity of 1.0000 indicates 100% purity. In all cases, the peak purity for SPC-100270 was 0.9999.

Figure 4 contains chromatographic traces of derivatized methanol (blank), SPC-100270, SPC-100580, and the racemate containing SPC-102860 and SPC-103980 at 330 nm. Solutions containing a mixture of SPC-100270 and these compounds were also derivatized and analysed. For chromatograms containing detectable peaks, the absorbance spectrum for each peak was compared to that of the reference standard SPC-100270 peak by means of the similarity index calculated by the SPD-M6A software. Identical absorbance spectra would have a similarity index of 1.0000. As can be seen in Fig. 4, no chromatographic peaks were observed in the blank beyond the solvent front. Table 2 contains retention times and similarity indices for the compounds studied. Based on relative retention times and the similarity indices, SPC-100270 may be distinguished from SPC-100580 on the basis of retention time and, when present, does not appear to affect the separation and quantification of SPC-100270. SPC-102860 also may be distinguished from SPC-100270 on the basis of retention time and when present, does not appear to affect the analysis of SPC-100270. However, it is not possible to distinguish between SPC-100270, SPC-103980, or a mixture of the two when assayed under the conditions described. No peak was detected at 330 nm for the process intermediate or the synthetic precursor.

Stressing underivatized SPC-100270 solutions with heat, heat and acid, or heat and base had no apparent effect on the analysis of SPC-100270. That is, no peaks other than the SPC-100270 peak were present in the chromatograms. The similarity index of the absorbance spectrum of the SPC-100270 peak to that of the SPC-100270 standard peak was 0.9997 or greater for all solutions.

The OPA-derivatized, peroxide-stressed SPC-100270 solution had one peak with an absorbance maximum at 203 nm and a retention time of 28.49 min. The similarity index of the absorbance spectrum of this peak to that of the SPC-100270 standard peak was 0.7942. The absence of an SPC-100270 peak in the chromatogram of the OPA-derivatized, peroxide-stressed SPC-100270 solution may be due to degradation of the SPC-100270 or to the degradation of, or reaction with, one or more of the derivatization reagents. It was not possible to determine from the results of this experiment which of these possibilities, if either, is correct. However, thin-layer chromatography experiments indicated that SPC-100270 is stable in the presence of 3% hydrogen peroxide.

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