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Stability studies and high-performance liquid chromatographic procedures for L-648,548 and its major degradates in an animal health formulation

Arthur J. Faulkner*, Qingxi Wang, Pierre DeMontigny, Joan S. Murphy

Merck Research Laboratories, P.O. Box 4, WP78-110, West Point, PA 19486-0004, USA

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Abstract

L-648,548 is a new avermectin which was evaluated for the development of an animal health formulation. A stability-indicating method for the assay of 5% (w/v) L-648,548 in an animal health formulation has been developed using reversed-phase high-performance liquid chromatography (HPLC) with UV detection (245 nm). The procedure to determine L-648,548 is linear and accurate over the range 80–120% of the target concentration with a limit of quantitation of 0.2%. Validation data are presented. Also, two related degradates of this compound were observed during the stability studies of the L-648,548 formulation. These degradates were determined to be the 2-epimer formed in the presence of base and the 8a-oxo degradate formed by oxidation. Identification of these compounds following direct chemical synthesis was based on mass, UV and NMR spectroscopy. The mechanistic pathways for the formation of these degradates are discussed. The 8a-oxo degradate has a modified chromophore, thus requiring a second HPLC method with detection at 280 nm that was also validated.

Keywords: High-performance liquid chromatography; Ivermectin; L-648,548; Stability; Degradates; Isomerization; Oxidation

1. Introduction

The avermectins are a class of therapeutically important macrolides that exhibit a broad spectrum of activity and very high potency towards both endo- and ectoparasites [1–4]. L-648,548 is an analog of ivermectin wherein the disaccharide group at the C-13 position is replaced with a methoxyethoxymethyl group. L-648,548 consists of two components in an approximately 9:1 ratio: an a-component with an ethyl substituent at the C-26 position and a b-component with a methyl substituent at this position. This compound was evaluated as the active ingredient in an anti-parasitic animal health formulation. Assays were needed to monitor drug concentration and degradate for-

* Corresponding author. Tel.: (215)652-2830; fax: (215)652-2821.

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mation in the formulation to establish product stability. The most commonly observed degradation reactions of ivermectin in formulations are acid-catalyzed cleavage of the disaccharide moiety, which yields the monosaccharide and aglycone [5], base-catalyzed formation of the 2-epimer [6] and oxidation at the C-8a position to yield the 8a-oxo degradate [7].

Because L-648,548 does not possess a disaccharide moiety, the major degradates for this compound were determined to be the 2-epimer and the 8a-oxo degradate (Fig. 1). This paper describes the use of reversed-phase high-performance liquid chromatography (HPLC) to achieve the separation of these compounds along with the required validation data to demonstrate the accuracy, precision and linearity of the method to determine L-648,548 and its 2-epimer degradate and of the separate method to determine the 8a-oxo degrate due to the modification of the chromophore. This paper also describes the synthesis, identification and reaction kinetics leading to the postulated mechanism for the formation of these degradates.

2. Experimental

2.1. Instrumentation

The liquid chromatograph consisted of a Shimadzu (Kyoto, Japan) LC-600 pump, a Shimadzu SIL-6B injector, a SYS-TEC (Minneapolis, MN, USA) column heater, a Shimadzu SPD-6AV detector and a Shimadzu CR501 integrator. The photodiode-array detector was a Hewlett-Packard (Waldbronn, Germany) Model HP 1040M. The chromatographic columns were a Zorbax (MAC-MOD Analytical, Chads Ford, PA, USA) RX-C18, (80 Å, 5 μm, 25 × 0.46 cm i.d.) and a Zorbax RX-C8, (80 Å, 5 μm, 25 × 0.46 cm i.d.).

Proton NMR spectra for L-648,548 were obtained with a Varian Unity Plus 500 MHz NMR spectrometer operating at 499.861 MHz. About 1 mg of sample was dissolved in 0.65 ml of CD_3OD (99.96 at.% D; Merck Isotopes). Proton chemical shifts were referenced internally to tetramethylsilane at 0 ppm. All spectra were acquired at 25°C. A two-dimensional absolute value COSY spectrum was acquired to assign chemical shifts; 32 transients were acquired on each of 256 increments. A sine-bell was applied in each dimension before Fourier transformation.

Proton NMR and COSY spectra for the 2-epimer were obtained with a Varian Unity 400 MHz NMR spectrometer operating at 399.953 MHz. Less than 1 mg of sample was dissolved in 0.65 ml of CD_3OD and the spectra were acquired using conditions similar to those for L-648,548. For the COSY, 128 transients were acquired on each of 256 increments. The NOESY data was obtained with a Varian Unity Plus 500 MHz NMR spectrometer operating at 500.153 MHz. The mixing time was 500 ms. There were 48 transients acquired on each of 224 increments. A phase-shifted sine-bell was applied in each dimension before Fourier transformation.

For the 8a-oxo derivative, proton 1D and COSY data were obtained on Varian Unity 400 spectrometer operating at 399.953 MHz. About 1 mg of sample was dissolved in 0.65 ml of CD_3OD and the spectra were acquired using conditions identical with those for the 2-epimer.

FAB mass spectra were obtained on a VG-7070 G mass spectrometer with magic bullet as the matrix. UV-visible spectra were obtained with a Hewlett-Packard HPLC with diode-array detection. pH measurements were taken with an Orion Model 710A pH meter (Orion Research, Boston, MA, USA).

2.2. Materials

HPLC-grade methanol and water and reagent-grade 0.2 M sodium hydroxide and 2 M hydrochloric acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ethyl alcohol USP (190 proof) was obtained from Quantum Chemical (Tuscola, IL, USA). Butylated hydroxytoluene (BHT) and L-648,548 were obtained from Merck Research Laboratories (Rahway, NJ, USA). The base and oxidation degradates of L-648,548 were prepared according to the published procedure with slight modifications [6]. Reagent-grade benzoic acid and sodium benzoate (99%) were obtained from Aldrich (Milwaukee, WI,

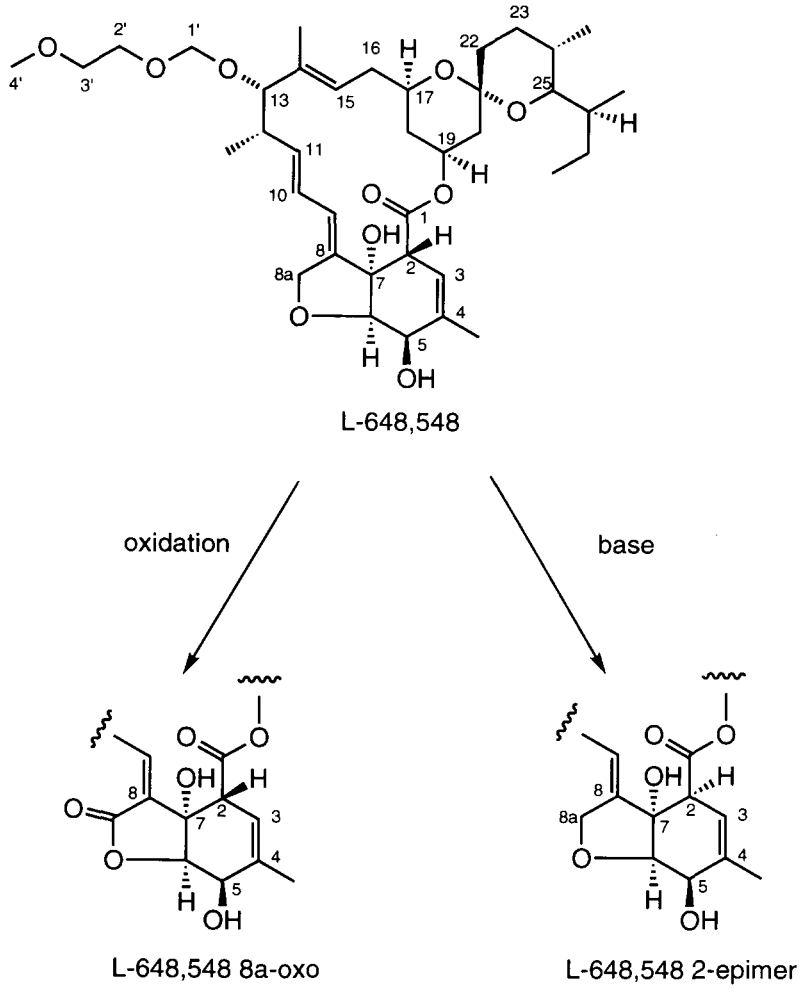


Fig. 1. Structure of the B1a component of L-648,548 and its major degradates.

USA). Denatonium benzoate was obtained from Macfarlan Smith (Edinburgh, UK). Denatonium saccharide (min. 95%) was obtained from Sigma (St. Louis, MO, USA).

2.3. 2-Epimer-L-648,548 synthesis

In a 100 ml round-bottomed flask, 50 ml of a 0.07 M solution of potassium hydroxide in 95% aqueous methanol was placed and, while protected under nitrogen and with constant stirring, L-648,548 (250 mg, 0.37 mM) was added in portions. This mixture was then stirred under nitrogen for 3 h. The reaction was quenched with 0.25 ml of acetic acid. The work-up involved the addition of 30 ml of saturated brine solution followed by extraction with methylene chloride three times. The combined methylene chloride layers were washed with saturated brine solution and water, dried over anhydrous magnesium sulfate and concentrated under vacuum to afford a crude mixture. Isolation by column chromatography using hexane-THF-CH_2Cl_2 (2:1:1, v/v/v) as the eluent afforded spectroscopicaly pure 2-epimer-L-648,548 with a yield of 14%.

2.4. 8a-Oxo-L-648,548 synthesis

A 10 ml volume of a methanol solution of 100 mg of L-648,548, 1.00 ml of *tert*-buty hydroperoxide and 0.107 g of copper (II) chloride were stirred at room temperature for 12 h. The reaction mixture was quenched by adding 20.0 ml of 2.0 mM sodium hydrogencarbonate solution, saturated with sodium chloride and then extracted with 50.0 ml methylene chloride. The combined organic layers were washed with saturated brine solution, dried with anhydrous magnesium sulfate and concentrated to give a yellowish oil. The 8a-oxo derivative was purified by preparative HPLC (mobile phase, acetonitrile–water (75:25, v/v); Zorbax ODS preparative column, 25 cm × 21.2 mm i.d.; flow-rate, 9.0 ml min^{-1}; detection, UV at 290 nm). The fractions with a retention time of 35.7 min were collected and concentrated under vacuum at about 30°C to afford spectroscopically pure 8a-oxo derivative as a white powder (8.3 mg, 8% yield).

2.5. Standard and sample preparation for L-648,548 and 2-epimer determination

The standard solution for L-648,548 was prepared in methanol to contain 250 μg ml^{-1}. The sample was prepared by transferring 0.5 ml of the 5% (w/v) L-648,548 formulation into a 100 ml volumetric flask and diluting to volume with methanol (concentration = 250 μg ml^{-1}).

2.6. Preparation of 2-epimer degradate solution

A 500 μl volume of 0.2 M NaOH was added to 500 μl L-648,548 standard solution and reaction was allowed to proceed for 2 min at ambient temperature. The reaction was quenched with 60 μl of 2 M HCl. A 20 μl volume was injected into the HPLC system within 1 h after preparation.

2.7. HPLC procedure for L-648,548 and 2-epimer determination

The mobile phase consisted of methanol–water (80:20, v/v) pumped at 1.0 ml min^{-1}. The column (Zorbax C-18 RX) was thermostated at 35°C with UV detection at 245 nm (0.08 aufs). The injection volume was 10 μl. The run time was 45 min.

2.8. Standard and sample preparation for 8a-oxo determination

BHT was prepared as the standard for 8a-oxo determination by diluting with methanol to a concentration of 2 μg ml^{-1}. The sample was prepared by transferring 1.0 ml of the 5% (w/v) L-648,548 formulation into a 50 ml volumetric flask and diluting to volume with methanol.

2.9. HPLC procedure for 8a-oxo determination

The mobile phase consisted of methanol–water (80:20, v/v) pumped at 1.0 ml min^{-1}. The column (Zorbax C-8 RX) was thermostated at 30°C with UV detection at 280 nm (0.08 aufs). The injection volume was 25 μl. The run time was 60 min.

3. Results and discussion

3.1. Stability of L-648,548

Investigations of the stability of L-648,548 revealed that the main degradates, the 2-epimer and 8a-oxo derivatives, are formed by base-catalyzed isomerization and oxidation, respectively. L-648,548 is not susceptible to acid-catalyzed hydrolysis based on kinetic data. The major degradate produced in alkaline solution is the 2-epimer, which is formed by isomerization at the C-2 position of the parent compound (Fig. 1). The reaction by which this degradate is formed has been described by Pivnichny et al. [6]. Similarly, addition of base (sodium hydroxide) to the formulation produced the 2-epimer degradate.

3.2. Identification and synthesis of 2-epimer degradate

The 2-epimer compound was synthesized by the treatment of L-648,548 with sodium hydroxide. The FAB mass spectrum showed the same molecular weight as the parent compound ($M^+ + H$ = 675). ^1H NMR data obtained for L-648,548 and

Table 1
^{1}H NMR data for L-648,548 and its 2-epimer and 8a-oxo degradates

	L-648,548	2-epimer-L-648,548	8a-oxo-L-648,548
C2-H	3.22 (m, 2.0)	3.18 (m, 2.6)	3.37 (m, 1.5, 2.5)
C3-H	5.42 (m, 1.6)	5.55 (m, 1.7, 2.6)	5.75 (m, 1.6, 2.5)
C4-CH_3	1.83 (3H, brd)	1.84 (3H, m, 1.3, 2.7)	1.88 (3H, t, 2.0)
C5-H	4.23 (brd)	4.28 (brd)	4.37 (d, 5.8)
C6-H	3.76 (d, 6.0)	4.00 (d, 2.6)	4.24 (d, 5.6)
C8a-H	4.64 (dd, 15.0, 2.7)	4.57 (dd, 13.3, 2.0)	–
C8a-H	4.57 (dd, 15.0, 2.7)	4.12 (dd, 13.5, 2.3)	–
C9-H	5.78 (dt, 11.4, 2.7, 2.7)	5.95 (dt, 11.2, 2.3, 2.3)	6.61 (d, 11.8)
C10-H	5.87 (dd, 15.0, 11.4)	5.77 (dd, 15.0, 10.0)	7.20 (dd, 15.2, 11.5)
C11-H	5.66 (dd, 15.0, 10.2)	5.60 (dd, 15.0, 10.1)	6.21 (dd, 15.3, 9.8)
C12-H	2.64 (m)	2.53 (m)	2.82 (m)
C12-CH_3	1.15 (3H, d, 7.3)	1.16 (3H, d, 7.0)	1.19 (3H, d, 7.0)
C13-H	3.96 (brd)	3.94 (brd)	4.03 (brd)
C14-CH_3	1.53 (3H, s)	1.55 (3H, s)	1.58 (3H, s)
C15-H	5.27 (t, 8.3, 8.3)	5.10 (brd, 8.7)	5.33 (m)
C16-H	2.28 (2H, m)	2.28 (2H, m)	2.31 (2H, m)
C17-H	3.70 (m)	3.71 (m)	3.75 (m)
C18-H_e	1.89 (brd, 12.9)	1.82 (m)	1.88 (obs)
C18-H_a	0.79 (m)	0.63 (q, 11.5)	0.87 (obs)
C19-H	5.00 (m)	5.32 (m)	5.15 (m)
C20-H_e	2.19 (ddd, 12.0, 4.5, 1.4)	1.82 (m)	2.12 (ddd, 11.9, 4.8, 1.5)
C20-H_a	1.19 (t, 11.7)	1.47 (m)	1.28 (m)
C22-H	1.63–1.50 (2H, obs)	1.66–1.52 (2H, obs)	1.65–1.50 (2H, obs)
C23-H	1.63–1.50 (2H, obs)	1.66–1.52 (2H, obs)	1.65–1.50 (2H, obs)
C24-H	1.56 (obs)	1.52 (obs)	1.55 (obs)
C24-CH_3	0.80 (3H, d, 6.6)	0.80 (3H, d, 5.8)	0.81 (3H, d, 5.3)
C25-H	3.24 (brd, 7.5)	3.24 (brd, 9.0)	3.25 (m)
C1′-H	4.71 (d, 6.6)	4.69 (d, 7.1)	4.74 (d, 6.9)
C1′-H	4.64 (d, 6.6)	4.63 (d, 7.1)	4.67 (d, 6,9)
C2′-H	3.86 (dt, 11.4, 4.2, 4.2)	3.86 (dt, 10.9, 4.7, 4.7)	3.86 (dt, 11.1, 4.6, 4.6)
C2′-H	3.65 (dt, 11.4, 4.2, 4.2)	3.65 (ddd, 11.0, 5.6, 4.9)	3.65 (dt, 10.9, 4.8, 4.8)
C3′-H	3.54 (2H, t, 4.8)	3.54 (2H, t, 4.8)	3.55 (2H, t, 4.7)
OCH_3	3.36 (3H, s)	3.36 (3H, s)	3.36 (3H, s)

Spectra were recorded in CD_3OD solution; chemical shifts are given in ppm relative to internal tetramethylsilane; splittings and coupling constants in Hz are given in parentheses. Abbreviations: s = singlet, d = doublet, t = triplet, m = multiple, brd = broad, a = axial, e = equatorial, obs = obscured.

its base-catalyzed degradate are summarized in Table 1. The chemical shifts for the C-2 hydrogens on both compounds are very close (δ 3.22 and 3.18 ppm), in agreement with avermectin 2-epimer (δ 3.22 ppm) [6]. However, there are substantial differences in chemical shift for the C6, C8a, C9, C19 and C20 hydrogens. The chemical shifts of the two C8a hydrogens are different, suggesting that they are not in the same environment. Also, the chemical shifts of these hydrogens have moved upfield and their chemical shift difference is much larger (δ 4.57 and 4.12 ppm) in comparison with L-648,548 (δ 4.64 and 4.57 ppm). Similar results have been reported for the C8a hydrogens of avermectin 2-epimer (δ 4.61 and 4.13 ppm) [6]. Overall, the ^{1}H NMR data in Table 1 indicate that the "lower part" of the L-648,548 structure is perturbed from this degradation reaction, which provides evidence of C2 epimerization. The chemical shift changes that were observed with L-648,548 2-epimer are in good agreement with the data reported for the base-catalyzed degradate of avermectin [6]. Additionally, the relative retention time of the 2-

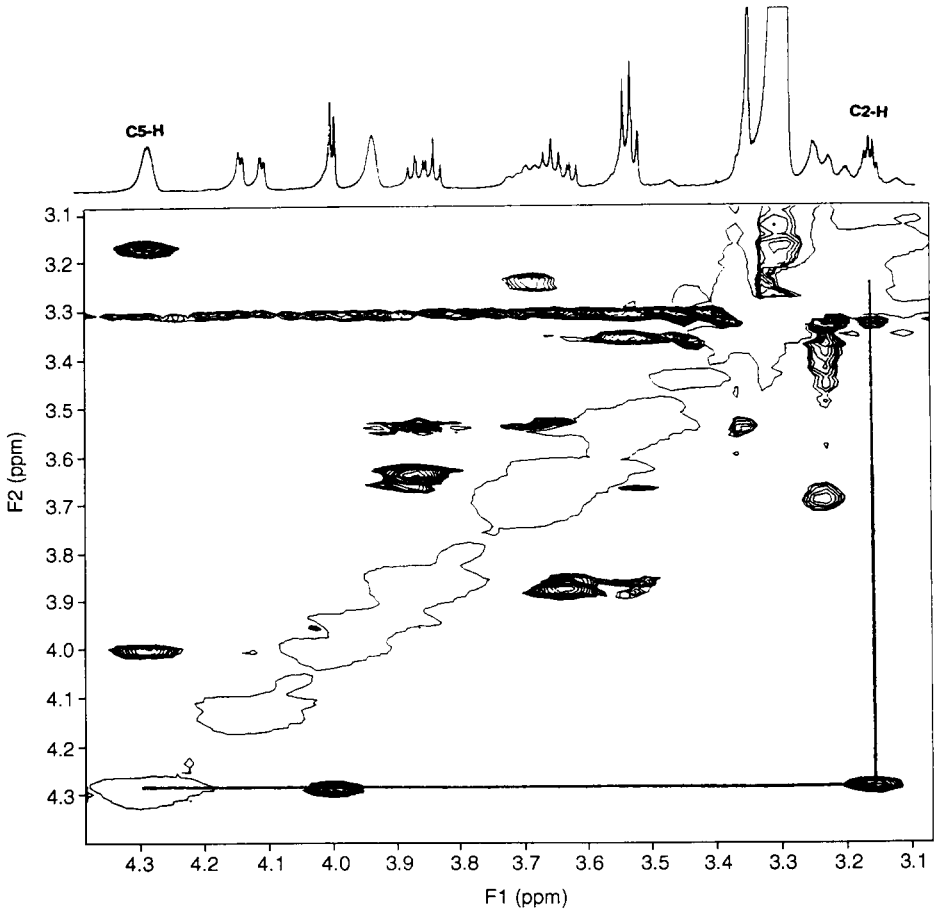


Fig. 2. NOSEY NMR spectrum of 2-epimer-L-648,548.

epimer (compared with the parent compound) was 1.22 for L-648,548 and 1.26 for avermectin [6] using reversed-phase HPLC conditions.

The structure of 2-epimer-L-648,548 was further confirmed by the NOESY experiment. The NOE spectrum shown in Fig. 2 clearly suggests very strong dipole–dipole interaction between the C2 and C5. With C2 and C5 hydrogens in the cis position as in 2-epimer-L-648,548, they are expected to be spatially close enough for an NOE to be observed. This evidence, combined with the relatively strong acidity of the C2 hydrogen, unambiguously confirmed the isomerization at the C2 position. Interestingly, no NOE was observed between the C2 and C6 hydrogens.

3.3. Determination of L-648,548 and 2-epimer

The method to determine L-648,548 and the 2-epimer in the formulation consists of dilution in methanol followed by HPLC analysis. Owing to the limited availability of pure synthetic degradate, area-% relative to L-648,548 B1a in the same sample is the preferred determination parameter for the 2-epimer. A typical chromatogram is illustrated in Fig. 3. Because the formulation is a solution that is diluted in methanol for the sample assay, the recovery of the 2-epimer is assumed to be quantitative (100%).

3.4. General base-catalyzed decomposition of L-648,548 and proposed mechanism

The 2-epimerization of ivermectin with sodium hydroxide has been documented by Pivnichny et al. [6]. Their investigations revealed that in addition to the formation of the 2-epimer in a strongly basic solution, the delta-2 isomer was also produced with disappearance of the 2-epimer. Epimerization is usually both specific and general base-catalyzed. This means that residual base present in the formulation, for example, could catalyze the isomerization of L-648,548. This was confirmed with a solution of L-648,548 in methanol stressed at 60°C in the presence of triethylamine. As indicated by HPLC analysis, the 2-epimer degradate was gradually formed with no delta-2 isomer (Scheme 1) observed after 12 h (Fig. 4). After several days at 60°C in the presence of triethylamine, low levels of the delta-2 isomer were observed at a relative retention time of 1.35 (in agreement with Pivnichny et al. [6]). No epimer was observed when L-648,548 was stressed without triethylamine present, suggesting that the 2-epimerization of L-648,548 is not thermally induced.

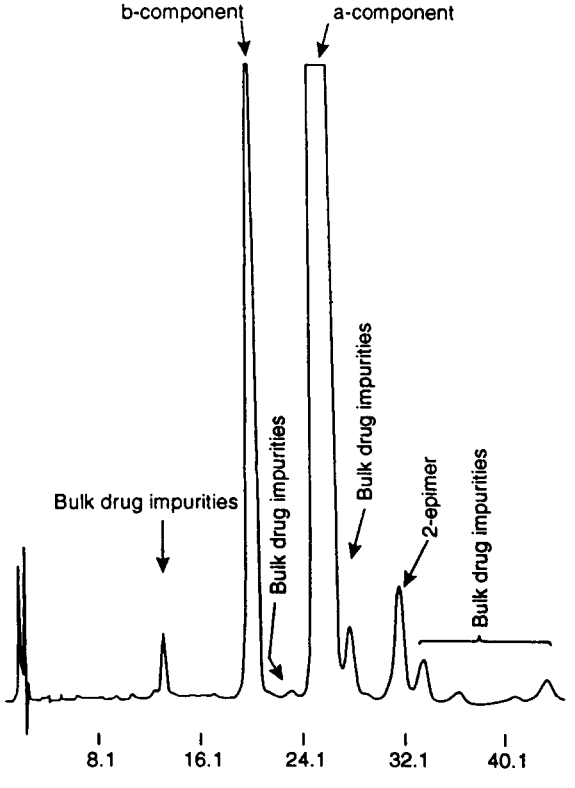
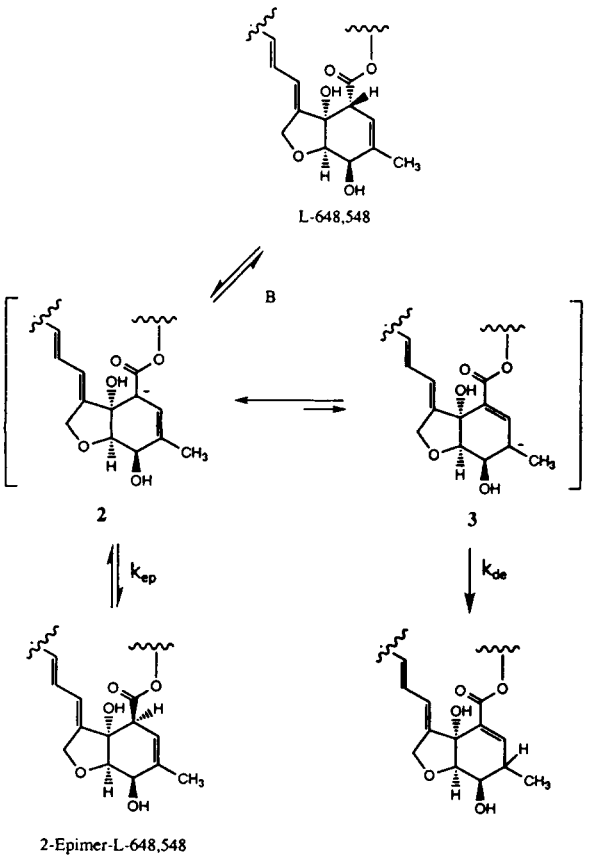


Fig. 3. Typical chromatogram of L-648,548 and 2-epimer-L-648,548.



Scheme 1. Base-catalyzed isomerization of L-648,548.

These results demonstrate that the rate of C2 epimerization is much greater than the rate of formation of the delta 2 isomer ($k_{ep} \gg k_{de}$). Similar results were reported by Pivnichny et al. [6]. These results can be explained by the formation of a stable carbanion intermediate **2**, which would then abstract a proton from the solvent or the C7 hydroxyl. Isomerization of **2** to the carbanion **3** would be kinetically unfavorable owing to the destabilizing effect of the C4 methyl group (Scheme 1).

Additional investigations were performed to explore the effect of added base such as a benzoate salt. Bitrex (denatonium benzoate) is an extremely bitter-tasing substance that can be added to a product to prevent accidental ingestion. Accelerated stability studies were conducted with Bitrex added to the L-648,548 formulation. Formulations containing 0.2% Bitrex, 0.06% sodium benzoate (equimolar to 0.2% Bitrex) and 0.06% benzoic acid were stored at 50, 60 and 75°C. The samples were analyzed by HPLC at 5, 12, 20 and 26 days and compared with control samples. Also,

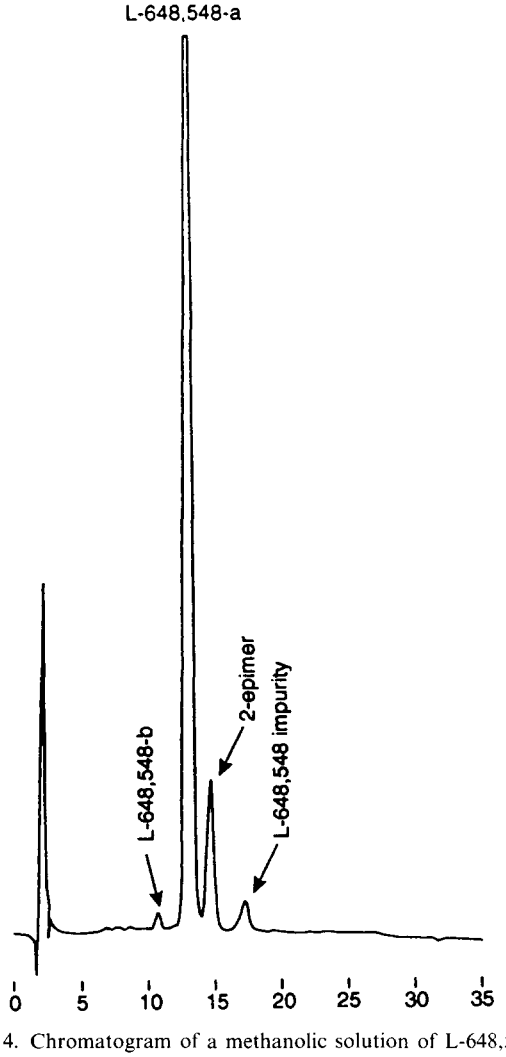


Fig. 4. Chromatogram of a methanolic solution of L-648,548 (0.15 M) stressed with triethylamine (0.01 M) at 60°C for 12 h.

investigations were conducted on the effect of adding 2 M HCl to the formulation with 0.2% Bitrex and with denatonium saccharide (0.2%) in lieu of denatonium benzoate.

Results from the studies with the formulation containing Bitrex or sodium benzoate are presented in Fig. 5. Clearly both solutions with additives were found to decompose more rapidly than the control sample, with 10% drug loss observed after 25 days at 75°C (compared with $<5\%$ in the control sample) with corresponding 2-epimer formation. These findings clearly suggest that the presence of the benzoate anion promotes drug decomposition in the formulation vehicle.

Based on these results, attempts were made to minimize the drug loss in the formulation with 0.2% Bitrex. The first attempt was to add benzoic acid to a formulation containing sodium benzoate (in place of Bitrex and at an equimolar concentration) to create a buffer in ethanol and lower the apparent pH. No amelioration was observed at either an equimolar concentration or a fourfold excess benzoic acid concentration, suggesting that ion dissocation in the formulation vehicle is significantly less than would be expected in aqueous solution ($pK_a = 4.2$ for benzoic acid). Apparent pH determinations indicated that benzoic acid was not effective in lowering the apparent pH of the formulation to that of the control (Table 2).

Further investigation involved the study of the effect of adding HCl to the formulation to obtain an apparent pH similar to that of the control sample. To achieve this, 40 μl of 2 M HCl were added to 20 ml of the formulation with 0.2% Bitrex. By adding HCl, the equilibrium between denatonium and benzoate would shift in favor of denatonium chloride association with benzoate converting to benzoic acid, which was found not to promote drug decomposition. Addition of HCl was found to prevent base-catalyzed decomposition in the formulation vehicle. No difference in stability was observed between the control sample and the formulation with 0.2% Bitrex and 2 M HCl.

Finally, an alternative denatonium salt was tested: denatonium saccharide at 0.2%. After 14 days at 75°C, there was less 2-epimer formed than in the control sample. This denatonium salt was

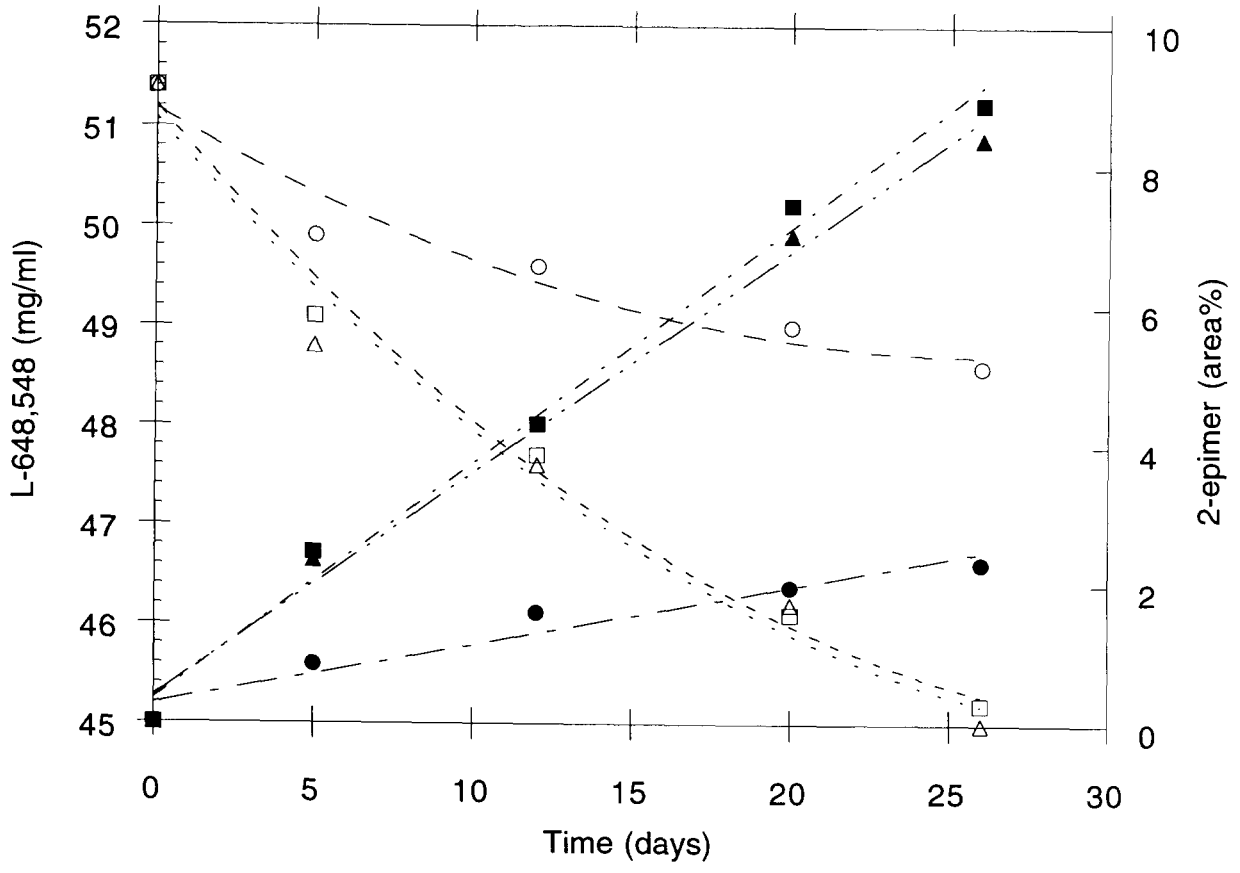


Fig. 5. Concentrations of L-648,548 (open symbols) and 2-epimer (filled symbols) versus time in the control formulation (circles), formulation with 0.20% Bitrex (squares) and formulation with 0.06% sodium benzoate (triangles) stored at 75°C.

found to prevent completely any of the base catalysis observed with Bitrex. Based on the results of these experiments, formation of the 2-epimer appears to be a general base-catalyzed reaction.

3.5. Identification and synthesis of 8a-oxo degradate

The oxidation of L-648,548 with *tert*-butyl peroxide using a transition metal ion (e.g. Cu^{2+}) as the catalyst rapidly generates the 8a-oxo degradate of L-648,548, which exactly matched that found in the aged or stressed formulations as indicated by HPLC. Pure 8a-oxo-L-648,548 was prepared using *t*-BuOOH–Cu^{2+} and its structure was assigned using several techniques. First, its molecular weight determined by mass spectrometry was 689 ($m^+ + H$), thus confirming the loss of two hydrogens and addition of an oxygen, which strongly suggests the oxidation of a methene ($-CH_2-$) to the corresponding carbonyl compound. Secondly, the UV–visible spectrum showed the maximum absorbance at 280 nm rather than 245 nm for the parent compound, indicating extended conjugation of the diene chromophore. Calculation according to Woodward–Fieser rules predicts an absorption maximum of 280 nm for the 8a-oxo degradate [8], in agreement with the experimental observation. Finally, the structure of 8a-oxo-L-648,548 was confirmed by proton NMR spectroscopy, which showed the lack of two C8a hydrogens and their allylic couplings with the C9 hydrogen (Table 1).

Table 2
Apparent pH of formulation with added Bitrex, sodium benzoate and benzoic acid

Sample	Apparent pH
Control formulation	6.1
Formulation + 0.2% Bitrex	7.2
Formulation + 0.5% Bitrex	7.7
Formulation + 0.6% sodium benzoate + 0.5% benzoic acid	7.1
Formulation + 0.6% sodium benzoate + 0.10% benzoic acid	6.9
Formulation + 0.6% sodium benzoate + 0.15% benzoic acid	6.8
Formulation + 0.2% Bitrex + 2 M HCl	<6.0

The oxidative degradation of avermectins has been studied using various oxidizing agents such as peroxides [2]. The peroxide-catalyzed oxidation of avermectin generates various degradation products with the formation of 8a-oxo as the predominant product. This compound is expected to be formed by a mechanism involving the peroxo intermediate as part of radical-initiated chain reaction [9–14]. To confirm such a mechanistic pathway, the 8a-hydroperoxide intermediate of L-648,548 (**4**) (Fig. 6) was isolated after L-648,548 had been oxidized with *tert*-butyl hydroperoxide in anhydrous methylene chloride. The structure was assigned based on the mass spectral and NMR data [spectral data for 8a-hydroperoxide of L-648,548: partial ^{1}H NMR (500 MHz, CH_3OD), δ 3.18 (1H, m, C2H), 5.42 (1H, m, C3H), 1.88 (3H, s, C4-CH_3), 4.35 (1H, brd s, C5H), 4.29 (1H, d, C6H), 6.09 (1H, d, C8H), 6.10 (1H, dd, C9H), 6.12 (1H, dd, C10H), 5.84 (1H, dd, C11H), 2.62 (1H, m, C12H), 3.96 (1H, brd s, C13H), 1.49 (3H, s, C14-CH_3), 5.18 (1H, brd s, C15H), 2.28 and 2.36 (2H, m, C16H), 3.69 (1H, M, C17H), 1.74 (1H, m, C18H_e), 0.81 (1H, q, C18H_a), 5.33 (1H, m, C19H), 1.96 (1H, ddd, C20H_e), 1.35 (1H, m, C20H_a); MS, *m/z* (707.5 M + H), 689.5 (M − OH), 673.5 (M − OOH)]. This 8a-hydroperoxide intermediate was also formed when L-648,548 was stressed by *tert*-butyl peroxide in methanol. However, after a prolonged reaction time, 8a-oxo-L-648,548 was formed as the final reaction product (Fig. 7).

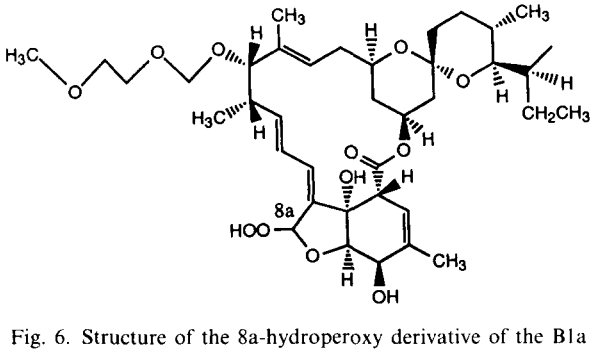


Fig. 6. Structure of the 8a-hydroperoxy derivative of the B1a component of L-648,548 (**4**).

The reaction of the 8a-hydroperoxide leading to the 8a-oxo product has not been fully characterized. However, in many cases, radical and metal ion-induced decomposition of secondary hydroperoxides is expected to yield both the lactone and lactol as shown in Scheme 2 [11,15]. Therefore, oxidation at the C8a position should result in the formation of at least some lactol (C8a-hy-

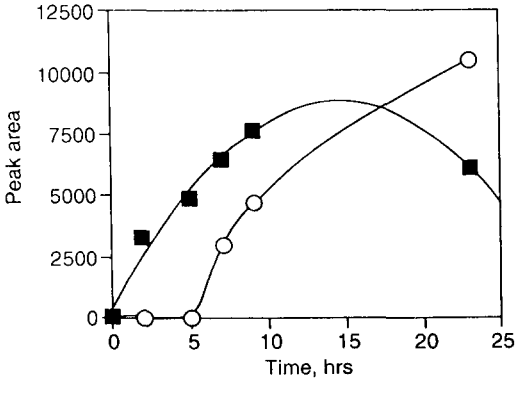
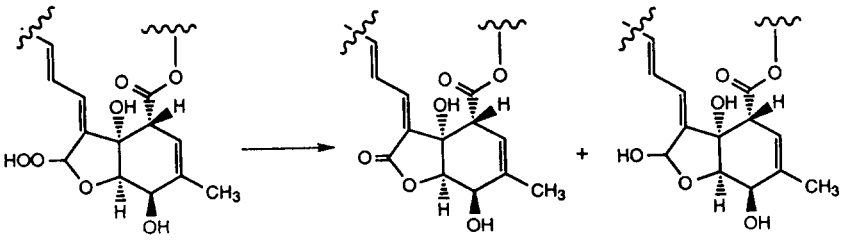


Fig. 7. Formation of 8a-hydroperoxide (squares) and the corresponding 8a-oxo (circles) degradates from the oxidation of L-648,548 (1.1×10^{-4} M) with *tert*-butylhydroperoxide (0.18 M) in methanol with copper(II) chloride (0.01 M) as a catalyst.



Scheme 2. Formation of 8a-oxo-L-648,548 B1a and 8a-hydroxy-L-648,548 B1a from the corresponding hydroperoxide.

droxy), but this product has not been detected in L-648,548 formulations.

3.6. Determination of 8a-oxo concentrations

The oxidation product 8a-oxo of L-648,548 (Fig. 1) was assayed to ensure that the antioxidant BHT added to the formulation is effective to prevent the oxidation of the drug in the formulation. This degradate was found in the formulation stressed with peroxide. Note that the bulk drug contains low impurity levels of 8a-oxo. Identification of this degradate was also confirmed by the injection of an authentic reference standard. In this case, however, this compound is nearly transparent at 245 nm, the analytical wavelength for assay as L-648,548. The absorption maximum for the 8a-oxo derivative was found to be at 280 nm, the same wavelength as used to determine the concentration of the antioxidant BHT in the formulation. Consequently, the 8a-oxo levels can be determined simultaneously with BHT using a separate analytical procedure. Only a limited amount of pure 8a-oxo was synthesized to determine the response linearity and solution stability. The assay method requires the use of BHT as the reference standard to determine the levels (weight %) of 8a-oxo relative to BHT using a relative response factor (RRF). This RRF was established with a synthetic 8a-oxo standard obtained with a purity of 83.9%.

The method to determine the concentration of 8a-oxo in the formulation requires the use of Zorbax C8 RX column and a mobile phase consisting of methanol–water (80:20, v/v). A typical chromatogram is illustrated in Fig. 8. Quantitation was accomplished by peak-height measurement. However, for bulk drug assay in other laboratories, an Inertsil C-8 column and a mobile phase consisting of acetonitrile–0.1% (v/v) perchloric acid (65:35, v/v) are used. Quantitation of the levels of 8a-oxo was accomplished by peak area. Using the latter procedure, the 8a-oxo/BHT RRF was determined to be 0.27 and 0.26 by two other laboratories (internal report from J.E. Gasman and J.R. Pindar, dated 14 June 1994, Merck Research Laboratories, Rahway, NJ). Determination of the RRF by peak area using the BHT method averaged 0.27 at 50–150% of the BHT standard concentration (2 μg ml^{-1}), in good agreement with the other two groups. However, because BHT was determined by peak-height meausurement, 0.51 was used as the RRF for the determination of 8a-oxo.

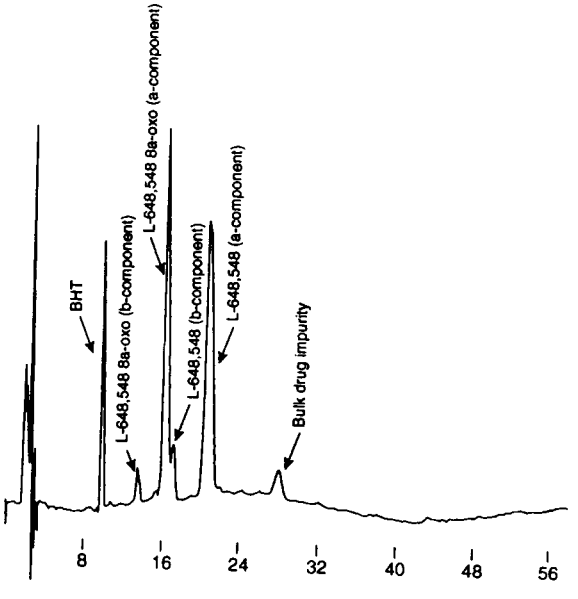


Fig. 8. Typical chromatogram of 8a-oxo-L-648,548 and BHT.

Table 3
Summary of accuracy data for L-648,548 determination

Spike	L-648,548 accuracy data (%)			
	Day 1	Day 2	Day 3	Average ($n = 9$)
80%	99.1	101.3	99.8	100.0
	99.7	100.5	100.8	(RSD = 0.85%)
	98.6	99.8	100.0	
90%	98.7	98.5	99.5	99.9
	99.5	99.1	101.7	(RSD = 1.09%)
	101.1	100.2	100.6	
100%	99.1	98.2	98.2	99.1
	98.9	100.5	98.4	(RSD = 0.79%)
	99.6	99.3	99.8	
110%	99.7	99.7	100.0	100.1
	99.9	100.6	100.8	(RSD = 0.48%)
	100.4	99.3	100.3	
120%	98.6	98.4	98.7	99.3
	99.6	100.2	100.1	(RSD = 0.64%)
	99.4	99.5	99.4	
Daily average ($n = 15$)	99.5	99.7	99.9	
RSD (%)	0.69	0.90	0.96	

3.7. Analytical method validation for the determination of L-648,548 and its degradates 2-epimer and 8a-oxo

3.7.1. Accuracy

The accuracy of the L-648,548 method was determined by adding L-648,548 at 80–120% of the theoretical 5% (w/v) potency (assay concentration 0.25 mg ml^{-1}) to the drug-free formulation. The accuracy determination was repeated on two additional days to determine the inter-day accuracy reproducibility. The results (Table 3) indicate that the daily recoveries averaged 99.5% (RSD = 0.7%, $n = 15$), 99.7% (RSD = 0.9%, $n = 15$) and 99.9% (RSD = 1.0%, $n = 15$) with an overall mean recovery of 99.7%. Single-factor ANOVA between the three groups resulted in a value of $F = 0.9$ ($F_{crit} = 3.2$, $\alpha = 0.05$), indicating that the variability of the data from the three days is not significantly different.

3.7.2. Precision

Method precision was demonstrated with six replicate determinations of L-648,548 in the formulation on three different days (Table 4). The daily averages were 5.02% (w/v) (RSD = 1.0%, $n = 6$), 4.96% (w/v) (RSD = 1.3%, $n = 6$) and 5.03% (w/v) (RSD = 1.5%, $n = 6$). The overall average of the assays is 50.1 mg ml^{-1} (100.2% of the label claim). Single-factor ANOVA between the three groups resulted in a value of $F = 2.1$ ($F_{crit} = 3.7$, $\alpha = 0.05$), indicating that the variability of the data from the three days is not significantly different.

Table 4
Summary of precision data for L-648,548 determination

	L-648,548 formulation assay (% w/v)		
	Day 1	Day 2	Day 3
	5.03	5.01	4.93
	4.94	4.89	5.05
	5.00	4.97	5.09
	5.08	4.88	5.12
	5.06	5.04	5.05
	5.02	4.98	4.95
Average	5.02	4.96	5.03
RSD (%)	0.97	1.26	1.53

For 8a-oxo determination, the RSD at a concentration of 2.0% was 0.1% ($n = 3$) and that at a concentration of 0.1% was 1.8% ($n = 3$).

3.7.3. Linearity

Linearity of the assay was evaluated using the accuracy data generated over three days (obtained by adding L-648,548 to placebo formulation samples), resulting in an average correlation coefficient $r = 0.9990$ (RSD = 0.06%). The slopes of these curves had an RSD of 5.39%. For degradate determination, the low-concentration linearity was checked by spiking placebo formulation with synthetically pure 2-epimer at concentrations of 0.1–3.1% (relative to the parent drug). A correlation coefficient $r = 0.9998$ was achieved.

The low-level response linearity of 8a-oxo-L-648,548 added to a placebo formulation was demonstrated at concentrations ranging between 0.1 and 2.0% (relative to the parent drug concentration). A correlation coefficient $r = 0.9999$ was achieved.

3.7.4. Standard and sample solution stability

L-648,548 standard and sample solutions kept for 72 h under laboratory ambient light and dark conditions showed excellent stability. No drug loss was observed during that period based on the absence of degradates and agreement of peak areas of the various 72 h samples with an RSD of 0.44%. A 1.5% solution of the 2-epimer added to a placebo formulation was examined under the same conditions, and all assay values were within 2.0% (RSD = 1.3%) of the original concentration.

Solutions of vehicle with 0.1 and 0.2% 8a-oxo degradate were assayed after 72 h in a freezer and at room temperature under laboratory light and dark conditions. There was no change in the 8a-oxo concentration for these samples. After 72 h, the samples containing 0.1% 8a-oxo degradate had an average assay of 101.1% (RSD = 2.22%) of the initial concentration, and samples containing 0.2% 8a-oxo degradate had an average of 100.1% (RSD = 0.51%) of the initial concentration.

3.7.5. Selectivity

No interference was observed for the determination of L-648,548 B1a and B1b in the formulation, as shown following the injection of a placebo formulation compared with the injection of a formulation sample. Drug substance impurities were also resolved from the parent components. The main degradate of L-648,548, the 2-epimer, was also resolved from the parent components. No interference is expected from the presence of the 8a-oxo compound because it is nearly transparent at the analytical wavelength used to determine L-648,548 and the 2-epimer (245 nm).

For the 8a-oxo determination, there was no interference with BHT or 8a-oxo from the formulation components.

3.7.6. Sensitivity

The sensitivity of the analytical procedure was determined by injecting low levels of the 2-epimer degradate. The limit of detection was 0.05% based on a signal-to-noise ratio (S/N) of 4. The limit of quantitation for the 2-epimer was 0.2% (RSD = 3.8%, $n = 3$).

Injection of 0.1% solution of 8a-oxo resulted in S/N = 69 and RSD = 1.8% ($n = 3$), thus providing excellent sensitivity. For this degradate, a limit of quantitation of 0.1% was desired, so lower levels were not injected.

4. Conclusions

Two rapid and sensitive stability-indicating analytical procedures for the assay of L-648,548 and its major degradates in an animal health formulation were developed. The method to determine L-648,548 was found to be precise, linear and accurate over the range 80–120% of the target concentration. The major degradates, 2-epimer and 8a-oxo, were identified by mass, UV and NMR spectroscopy, and can be accurately quantitated at low levels (0.1–0.2%). The results from accelerated stability studies clearly indicated that the formulation with added Bitrex undergoes base-catalyzed isomerization owing to the presence of the benzoate anion. The stability of L-648,548 in the formulation with added Bitrex was

improved significantly with the addition of HCl. Alternatively, the use of denatonium saccharide in lieu of Bitrex eliminated the formation of 2-epimer. The mechanistic pathways investigated in this stability study of L-648,548 should help in understanding the complex degradation reactions of other avermectin analogs.

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