Synthesis and Identification of the Primary Degradation Product in a Commercial Ophthalmic Formulation Using NMR, MS, and a Stability-Indicating HPLC Method for Antazoline and Naphazoline

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Abstract □ HPLC analysis of an anti-infective ophthalmic solution (Albalon-A), containing the active drugs naphazoline and antazoline, revealed a degradation peak of unknown identity. To elucidate the identity of the degradant, the active drugs were each hydrolyzed by refluxing at high pH, and their respective hydrolysis products were isolated and spectrally characterized by NMR, FT-IR, and MS for conclusive structure elucidation. The degradant's identity was confirmed by HPLC-MS analysis of Albalon-A ophthalmic solution to be the antazoline hydrolysis product *N*-[(*N*-benzylanilino)acetyl]ethylenediamine (**IV**). A stability-indicating HPLC method was then developed which was able to resolve **IV** from the active drugs. This HPLC method was then validated for quantitating the active drugs and **IV**. Validation studies demonstrated linear UV response at 280 nm, recovery > 98%, good reproducibility, and a detection limit of 2 μg/mL **IV**. Overall, the data demonstrated that the HPLC method was quantitative and specific for antazoline, naphazoline, and **IV**. Analysis of an expired stabilitry lot of the ophthalmic solution indicated the concentration of **IV** was 0.002% (w/v).

Introduction

Albalon-A ophthalmic solution is used to treat an inflammatory condition of the mucus membrane covering the anterior surface of the eyeball and the eyelids known as conjuctivitis or "pinkeye". Conjuctivitis can be caused by a host of conditions potentially leading to ocular irritation, such as allergy, dust, glare, chemicals, and bacterial infection. The active drug components in Albalon-A are naphazoline hydrochloride (a vasoconstrictor and decongestant) and antazoline phosphate (an antihistamine). The structures of these drugs are given in Figure 1 with their potential hydrolysis pathways.

This work was initiated in order to identify a degradant peak detected in several expired stability lots of Albalon-A ophthalmic solution. Rather than laboriously separate and isolate the unknown peak via preparative HPLC for spectral identification, it was decided to exploit the known chemistry of naphazoline, which has been shown to be susceptible to base-catalyzed hydrolysis at the carbon atom of the imidazoline functional group.[1,2] This hydrolysis results initially in the formation of the corresponding 1,2-diamine (**III** and **IV** in Figure 1), and potentially to the corresponding carboxylic acid (**V** and **VI**). It was believed that hydrolysis would be the primary degradation pathway of antazoline and naphazoline and that their hydrolysis products would be the most likely compounds potentially detected as the unknown degradant peak in Albalon-A.

During this work it was discovered that the hydrolysis kinetics of naphazoline was documented in the literature;[1,2] however this was not found to be true for the drug substance antazoline. Although analysis methods involving antazoline have been published,[1,3,4] no reports could be found describing the stability characteristics of this drug. As shown in Figure 1, antazoline also possesses an imidazoline functional group and we suspected that its hydrolytic chemistry may mirror that of naphazoline. Since the unknown degradant peak detected in Albalon-A could be from either antazoline or naphazoline, we attempted to synthesize all hydrolysis products of these drugs on the basis of the hydrolysis procedure for naphazoline described by Schwartz et al.[1]

This report describes the synthesis, isolation, and characterization of the hydrolysis products for both naphazoline hydrochloride and antazoline phosphate. In addition, the isolated hydrolysis products were used to identify the primary degradation component in an actual stability lot of Albalon-A ophthalmic solution and in antazoline phosphate raw material. To the authors' knowledge, this is the first report describing the hydrolytic synthesis of antazoline's primary degradation product and its subsequent identification in a pharmaceutical formulation. In addition, this report appears to be the first publication, using synthesized degradants, to prove assay specificity for the simultaneous quantitation of both antazoline and naphazoline in a pharmaceutical product.

Experimental Section

Materials—Antazoline phosphate and naphazoline hydrochloride were used as received from Allergan Pharmaceutical Science Operations, Irvine, CA, their certificates of analysis indicating purity values of 99.6% and 98.6%, respectively. These drugs were manufactured by CIBA Pharmaceuticals with lot numbers of 39623 and CMS-87-060-001 for antazoline and naphazoline, respectively. Albalon-A ophthalmic solution, stability lot #EH4272 (manufactured 1986, expired 1990) was received from Allergan Ireland through the Allergan Irvine Regulatory Dept. This sample was stored at room temperature for 5 years (in an ongoing stability program) prior to our analysis and contained degradant levels representative of several other product lots of the same age. Methanol (J. T. Baker Inc., Phillipsburg, NJ), heptane sulfonic acid, sodium salt (Eastman Kodak Co., Rochester, NY), and chloroform (Burdick and Jackson, Muskegon, MI) were HPLC grade. Concentrated phosphoric acid, glacial acetic acid, concentrated hydrochloric acid (Mallinckrodt, American Scientific Products, McGaw Park, IL), triethylamine (Eastman Kodak), and sodium hydroxide pellets (J. T. Baker) were reagent grade and used as received. All water used was filtered through a Milli-Q water purification system (Milli-Pore Inc., Milford, MA).

Instrumentation—*HPLC Analysis*—HPLC work was performed using a Waters 600E low-pressure mixing gradient solvent delivery controller and pump connected to a Waters 715 ultra-WISP autoinjector. Detection was by a Waters 490 multichannel variable ultraviolet detector (Waters Associates, Milford, MA) connected to a PE Nelson Series 900 A/D converter interfaced to a VAX 6210 computer (Digital Equipment Corp., Merrimack, NH) running PE Nelson Access*Chrom

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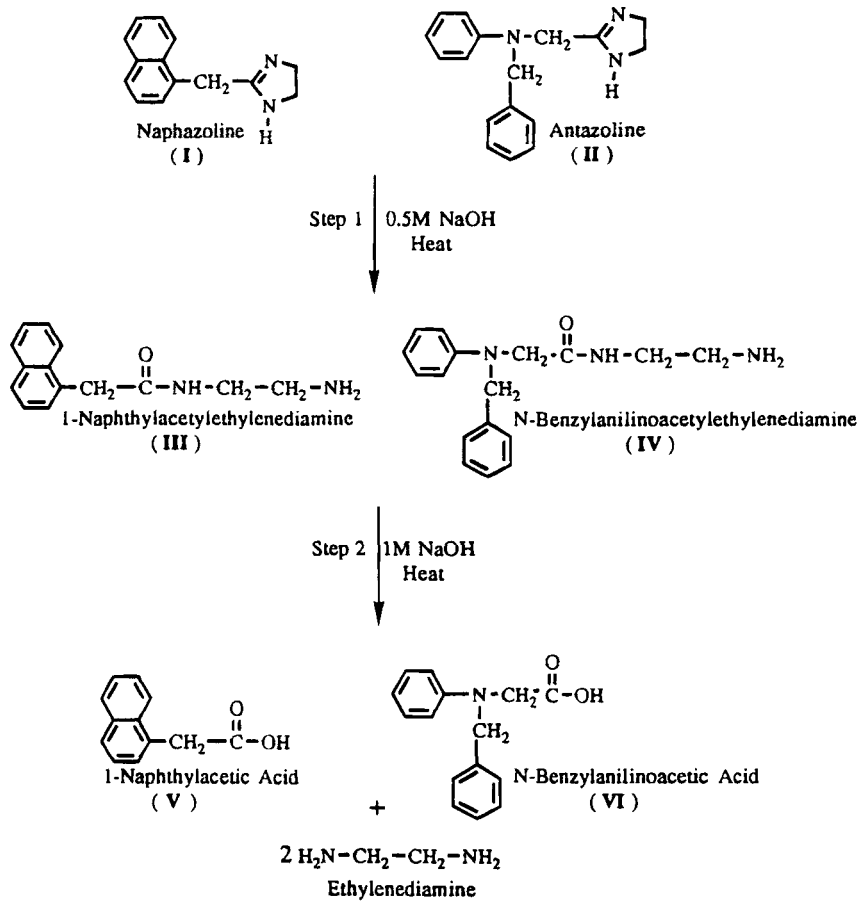

Figure 1—Structures of the active drugs in the ophthalmic solution with the potential hydrolysis path for each.

software, version 1.6 (PE Nelson Inc., Cupertino, CA) for data processing. Samples were analyzed by one of two HPLC methods.

HPLC Method 1—Method 1 used a mobile phase of 50:50 (MeOH:H_2O) containing 5 mM heptanesulfonic acid and 2% glacial acetic acid pumped at 1 mL/min through a Waters μ-Bondapak C_{18} column with UV detection at 280 nm. Sample and standard preparation was identical to HPLC method 2.

HPLC Method 2—Method 2 consisted of injecting 20 μL of sample into a 57:43 (MeOH:H_2O) mobile phase containing 22 mM heptanesulfonic acid, 0.1% dibutylamine, and 1% acetic acid pumped at 1 mL/min through a Beckman Ultrasphere C_{18} column (250 × 4.6 mm, 5 μm particles) with detection at 280 nm. Calibration standards of **I** and **II** were prepared at 50 and 500 μg/mL, respectively, in water. Sample preparation consisted of a 1:10 dilution of the ophthalmic solution with water using class A volumetric glassware. The concentration of **IV** determined in the stability sample was obtained using the synthetic **IV** reference standard. Selected chromatographic runs were coupled, in-line, to a Hewlett-Packard 1040A diode array detector to capture analyte UV spectra. See chromatograms for specific parameters and conditions used for analysis.

NMR Spectroscopic Analysis—NMR work was performed on a Varian XL-300 spectrometer (Varian Inc., Palo Alto, CA) running Varian version 6.1 NMR software, revision E. The synthesized degradants were dissolved in $CDCl_3$ (>99.8% isotopic purity, MSD Isotopes, Merck and Co., Rahway, NJ). ^1H NMR spectra were acquired using a frequency of 299.9 MHz, spectral width of 4000 Hz, acquisition time of 3.7 s, and pulse width of 32° collecting 64 repetitions at ambient temperature. ^{13}C NMR spectra were obtained similarly, except under proton decoupled conditions using a frequency of 75.4 MHz, acquisition time of 2 s, and collecting 2.0×10^5 repetitions. Data are reported as follows: chemical shift (in δ values or ppm), multiplicity (s = singlet, d = doublet, t = triplet m = multiplet), and assignment.

FT-IR Spectrophotometric Analysis—FT-IR spectra of the synthesized degradants were obtained using a Bio-Rad infrared spectrophotometer incorporating a FTS 15/80 optics bench and Bio-Rad 3200 data system running DDS 3200 software, version 4.3.1 (Bio-Rad, Digilab Division, Cambridge, MA). Diffuse reflectance internal reflectance FT-IR (DRIFTS) spectra were obtained by mulling the sample (**III** and **V**) in dry KBr, using a Bio-Rad diffuse reflectance accessory with beam aperture of 18 and resolution of 8, and acquired using 64 scans. The spectrum of **IV** (an oil) was generated similarly, except using a neat sample between sodium chloride blocks with a 0.1 mm cell path distance.

MS Analysis—Direct probe MS analysis of the synthesized degradants was carried out on a VG 7070E spectrometer in the low-resolution mode using electron impact ionization with an accelerating voltage of 70 eV. Samples were dissolved in HPLC grade methanol prior to evaporation on the direct insertion probe filament. Data was acquired using a 3 s/decade scan ranging from 50 to 600 Da.

HPLC-MS Analysis—HPLC-MS analysis was performed using a Shimadzu gradient HPLC interfaced to a Sciex API III triple stage quadrolpole mass spectrometer scanning from 180–400 Da at 4.1 s/scan in the positive ion mode. An atmospheric chemical ionization source (APCI) was used, which resulted in the detection of primarily molecular ion species. A Beckman Ultrasphere C_{18} HPLC column (250 × 4.6 mm, 5 μm particles) was used to run a linear mobile phase gradient of 30:70 (acetonitrile:20 mM ammonium acetate

buffer) to 90:10 (acetonitrile:20 mM ammonium acetate buffer) over 30 min at a flow rate of 1 mL/min.

Spectroscopic Data—*N-(1-Naphthylacetyl)ethylenediamine (III)*—UV (method 2 mobile phase) λ_{max} 248.0 nm; IR 3353 (s, NH_2), 3307 (s, NH_2), and 1642 (s, amide C=O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 0.5 (s, 2H, NH_2), 2.6 (t, 2H, CH_2), 3.2 (t, 2H, CH_2), 4.0 (s, 2H, CH_2), 5.8 (s, 1 H, NH), 7.4–8.0 (m, 7H, Ar-H); ^{13}C NMR ($DCCl_3$) ppm 41.2 (CH_2), 42.0 (CH_2), 42.5 (CH_2), Ar-C; 123.5, 125.5, 126.2, 127.0, 128.4, 128.6, 128.8, 131.2, 132.0, 134.1, 172.1 (C=O).

N-[(N-Benzylanilino)acetyl]ethylenediamine (IV)—UV (method 2 mobile phase) λ_{max} 248.0 nm; IR 3307 (s, NH_2), 3100–2800 (m, NH_2), 1669 (s, amide C=O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.2 (s, 2H, NH_2), 2.6 (t, 2H, CH_2), 3.2 (t, 2H, CH_2), 3.9 (s, 2H, CH_2), 4.6 (s, 2H, CH_2), 6.8–7.8 (m, 7H, Ar-H); ^{13}C NMR ($DCCl_3$) ppm 17.8 (CH_2), 32.2 (CH_2), 34.0 (CH_2), 36.0 (CH_2), Ar-C; 115.8, 116.2, 124.0, 128.1, 129.2, 131.1, 131.4, 132.0, 134.2, 134.4, 136.0, 146.4, 150 (C=O).

1-Naphthylacetic Acid (V)—UV (method 2 mobile phase) λ_{max} 248.0 nm; IR 2800–3500 (OH), 1740 (s, C=O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 3.45 (s, 4H, CH_2), 4.1 (s, 2H, CH_2), 7.0–8.2 (m, 7H, Ar-H); ^{13}C NMR ($DCCl_3$) ppm 39.0 (CH_2), Ar-C; 123.8, 125.8, 126.0, 126.8, 128.0, 128.2, 129.0, 130.1, 132.1, 134.0; 178.5 (C=O).

Synthesis and Isolation of Hydrolysis Products—The hydrolysis of antazoline and naphazoline was performed in the same manner as that described by Schwartz et al.[1] This consisted of refluxing each drug separately for 30 min in 0.5 M NaOH to reach step 1 of the hydrolysis. Another experiment separately refluxed each drug in 1 M NaOH for 2 h in an attempt to reach step 2 (see Figure 1). It was discovered that the hydrolysis of antazoline through step 1 was incomplete after 30 min in 0.5 M NaOH and therefore was refluxed an additional 30 min, whereupon it was fully converted to **IV**. Reaction completion was monitored by HPLC method 2. The hydrolysis products were recovered by cooling the basic reflux solutions, acidifying with concentrated HCl, and extracting into chloroform. The chloroform extract for each was then rotary evaporated down to several milliliters and separately dispersed onto glass plates prior to drying and scraping the residues; these were then subsequently stored in Teflon-sealed LC vials at −20 °C prior to NMR and MS characterization.

Results and Discussion

Characterization of Isolated Hydrolysis Products—Initially, during step 1 of the synthesis, the isolated hydrolysis products **III** and **IV** were analyzed by HPLC method 2 and found to be 99.1% and 99.7% pure (by area), respectively. The second phase of the synthesis, which attempted to hydrolyze antazoline and naphazoline through step 2 using more aggressive hydrolysis conditions,[1] also yielded samples which were analyzed by HPLC. The analysis indicated that only naphazoline hydrolyzed through step 2, to **V** (99.4% pure by area), and that antazoline had reacted only through step 1, to **IV**. A full spectroscopic characterization of each hydrolysis product was then undertaken to conclusively prove the identity of each isolated compound.

Hydrolysis products **III-V** were initially analyzed by low-resolution direct probe MS. The electron impact mass spectrum of **III** (Figure 2a) shows that the highest mass detected was 228 Da, which corresponds exactly to the molecular weight of **III** in Figure 1. In addition, the mass of each of the lower MW fragment ions in Figure 3a all correspond, in mass, to fragments of the **III** molecule. Similarly, the mass spectrum of **IV** (Figure 2b) indicates a probable molecular ion at 283 Da, which corresponds to the mass of **IV**. The smaller mass fragments in Figure 2b also are consistent with portions of the **IV** structure given in Figure 1. Figure 2c gives the MS spectrum of **V** which displays a relatively strong molecular ion at 186 Da, corresponding to the mass of **V**. Upon inspection of the data it was concluded that the MS spectra obtained for the isolated hydrolysis products were consistent with the proposed structures depicted in Figure 1.

The hydrolysis products were also characterized using both ^1H and ^{13}C NMR. The ^1H NMR spectroscopic data are provided in the Experimental Section. It is evident upon inspection of the spectral data that the chemical shift patterns and proton integration values obtained are consistent with the structures for **III-V**. The ^{13}C spectra obtained were also consistent with the structures in that the correct number of aromatic and aliphatic carbon signals for each of the hydrolysis products were observed.

FT-IR spectra were also obtained for the compounds which indicated that the correct functional groups were present for each of the hydrolysis products. Compounds **III** and **IV** both exhibited a strong amide carbonyl signal near 1669 cm^{-1} as well as primary amine absorption at 3307 cm^{-1}, indicating the presence of these functional groups. In contrast, compound **V** showed a typical carbonyl absorption at 1670 cm^{-1} and no sharp band for primary amine. Only compound **V** exhibited the expected broad absorption from 2500 to 3200 cm^{-1}, characteristic of carboxylic acids.

Overall, if one considers the aggregate of various spectral data gathered, it can be concluded that the isolated hydrolysis products yielded spectra consistent with the structures given in Figure 1. Further supporting evidence is provided by the synthetic route utilized, which has previously been shown to yield naphazoline hydrolysis products.[1] An interesting finding from this synthetic work was that while full hydrolysis of naphazoline had been accomplished (through step 2), antazoline was only hydrolyzed to **IV**. The tendency of antazoline to resist full hydrolysis suggests that naphazoline may be more likely to degrade to its acetic acid analog in an ophthalmic product.

HPLC Analysis of Albalon-A and Isolated Hydrolysis Products—Initially, an expired stability lot of Albalon-A was chromatographed using HPLC method 1, as had previously been done during the first observation of the unidentified peak. The ophthalmic solution was then subdivided and separately spiked with **III**, **IV**, and **V** whereupon it was discovered that the retention time of **IV** exactly coincided with that of the unidentified peak. Figure 3, parts a and b, gives the chromatograms of Albalon-A and Albalon-A spiked with **IV**, respectively. These chromatograms indicate that the unknown peak observed in the tail of antazoline (for the stability lot) appears to be the antazoline hydrolysis product **IV**.

Compound **IV** was also identified, at trace levels, in pure antazoline phosphate raw material. Detection of **IV** in raw material was possible only by injecting a concentrated 0.3 mg/mL solution of antazoline. Identification of **IV** was accomplished through the use of a diode array detector (DAD) coupled in-line using HPLC method 2. It was discovered that the overlaid UV spectra of the synthesized **IV** hydrolysis product, the suspect trace peak in antazoline raw material, and the unidentified peak in Albalon-A were all identical, as were their chromatographic retention times. These UV spectra and identical retention times observed provided additional evidence that the unknown degradant peak observed in Albalon-A originated from antazoline, and was **IV**.

Since HPLC method 1 did not adequately resolve **IV** from antazoline, HPLC method 2 was developed which provided superior selectivity, allowing all isolated hydrolysis products and parent drugs to be resolved from each other. Figure 4a shows the chromatogram obtained using method 2 for Albalon-A spiked with the synthesized hydrolysis products. Figure

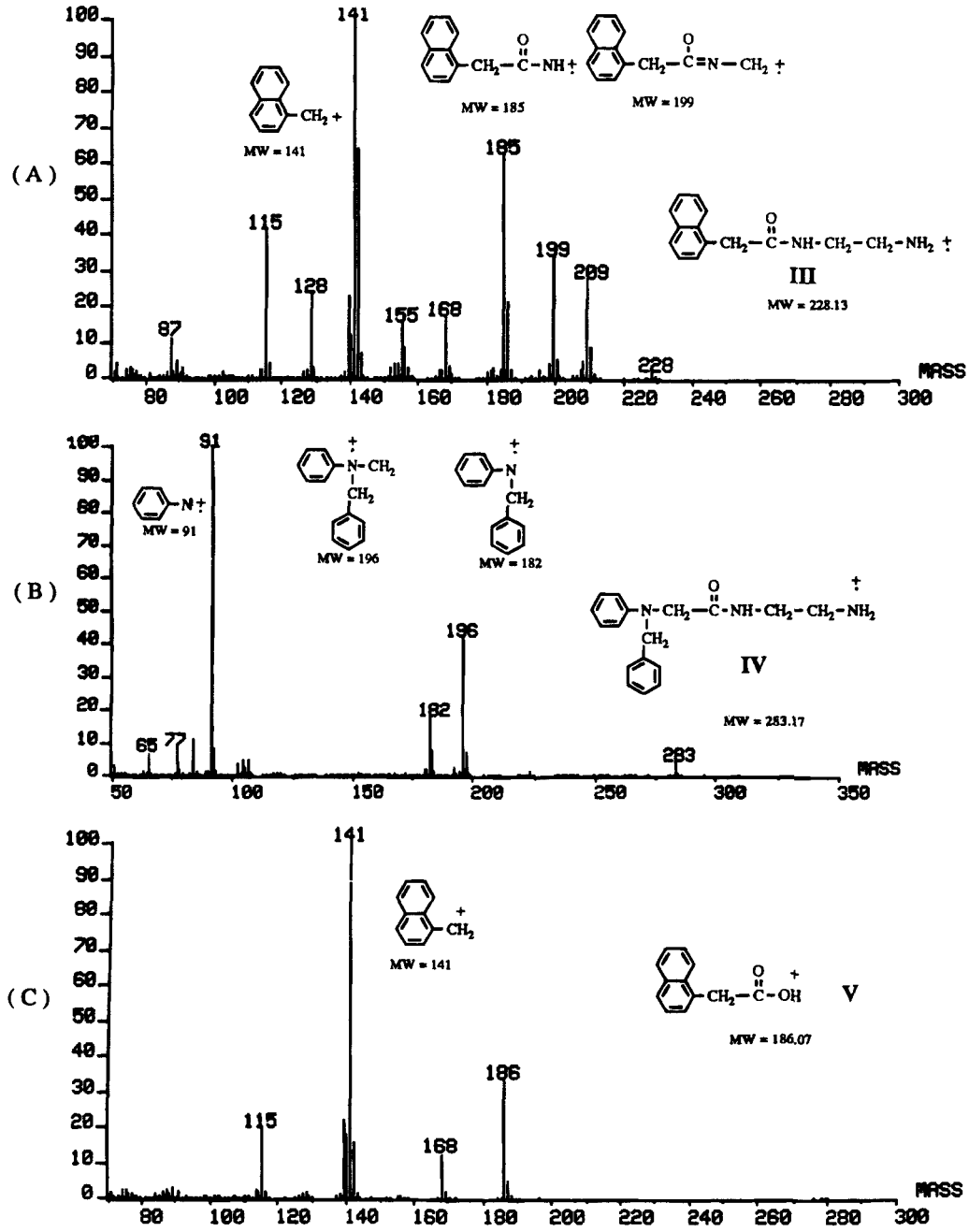

Figure 2—Electron impact mass spectra of synthesized (a) III, (b) IV, and (c) V.

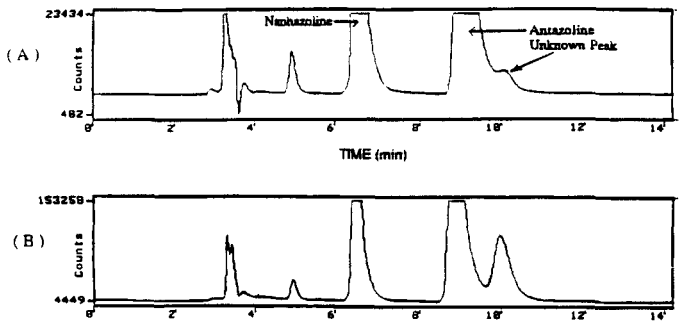

Figure 3—Chromatogram obtained using HPLC method 1 for (a) expired stability lot of ophthalmic solution and (b) expired stability lot of ophthalmic solution spiked with synthesized IV.

4b shows the chromatogram of the unspiked product. It is evident in this chromatographic system that **IV** and the prominent unknown peak at 32 min in Albalon-A again have identical retention times. These data further support the conclusion that the prominent unidentified peak in the ophthalmic solution is the initial hydrolysis product of antazoline, *N*-[(*N*-benzylanilino)acetyl]ethylenediamine (**IV**).

Since the acetic acid analog of antazoline (**VI**) could not be synthesized, even under more severe hydrolysis conditions,[1] **VI** would not be expected to form under normal product storage conditions. As Figure 1 indicates, formation of the fully hydrolyzed acetic acid analog of either naphazoline or antazoline results in the release of ethylenediamine, which would be a safety concern if complete hydrolysis were to occur. Therefore, a significant finding of this work was that no **V** was detected in the ophthalmic formulation studied.

Through the use of synthesized hydrolysis products, HPLC method 2 was proven to be the superior HPLC method investigated. This method was validated as the new stability-indicating analysis procedure for ongoing Albalon-A stability

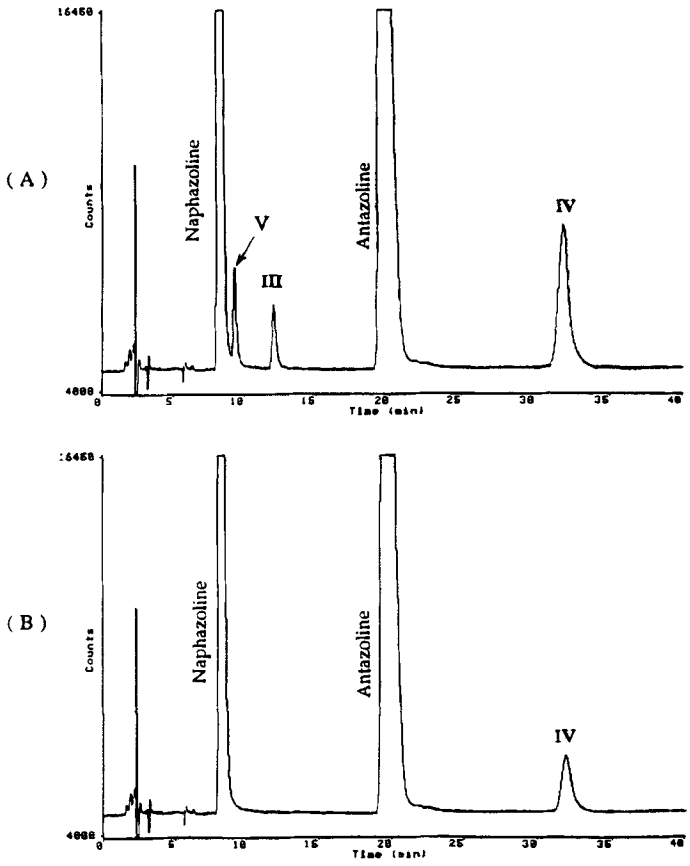

Figure 4—Chromatogram obtained using HPLC method 2 of (a) ophthalmic solution spiked with synthesized III-V and (b) expired stability lot of ophthalmic solution.

studies, due to its ability to resolve and quantitate the active drugs and their associated degradants.

For the conclusive determination of structure for the product degradant **IV**, Albalon-A and a standard solution of synthesized **IV** were separately analyzed using HPLC–MS as described in the Experimental Section. A gradient HPLC method was used due to the incompatibility of ion pairing reagents and buffering salts with the mass spectrometer. The degradation peak **IV** in Albalon-A and the synthetic **IV** standard gave identical chromatographic retention times using this gradient HPLC method, as shown in Figure 5. The ion chromatogram obtained for the HPLC–MS analysis of the synthetic **IV** standard yielded a large mass peak (M + H) at 284 Da which corresponded to the molecular weight of 283 (M+) previously obtained by direct probe MS (Figure 2b). Subsequent HPLC–MS analysis of Alablon-A (monitoring mass 284) yielded a peak of identical retention time to that of the synthetic **IV** standard. The mass spectrum obtained for the degradant (**IV**) in Albalon-A was then compared to the spectrum obtained for the synthetic **IV** standard peak (see Figure 6, parts a and b, respectively). The mass fragments detected are all identical, except for the mass 211 Da fragment in the stability sample. The 211 Da fragment is most probably a formulation component since complete resolution of all sample peaks (as shown in Figure 5) was not achieved during the HPLC–MS analysis. This is attributed to the different HPLC systems which were used to develop the gradient method and that which was interfaced to the mass spectrometer. Since **IV** could be selectively detected by monitoring the ion chromatogram obtained at 284 Da, further attempts to chromatographically resolve **IV** from formulation components were not pursued during the HPLC–MS analysis. Overall, the mass spectra and identical retention obtained for the

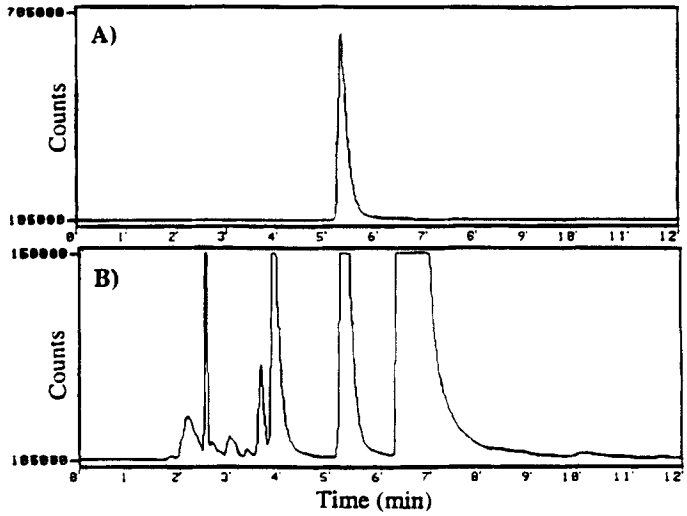

Figure 5—Chromatogram obtained (UV detection at 210 nm) using a gradient HPLC–MS method for analysis of (a) synthetic **IV** standard and (b) expired stability lot of ophthalmic solution.

Table 1—Recovery Values Obtained Using HPLC Method 2 ($n = 6$ Replicates)

Analyte	% Recovery			
	Day 1	Day 2	Day 3[a]	Average % ± RSD
Antazoline (0.5% (w/v)	99.5	97.8	99.1	98.8 ± 0.9
Naphazoline (0.05% w/v)	98.7	97.7	98.2	98.2 ± 0.5
Degradant IV (0.002% w/v)	101.1	103.2	96.2	100.2 ± 3.6

[a] Different analyst.

degradant in Albalon-A and synthesized **IV** indicate that they are the same molecular entity.

Validation of HPLC Method 2—Compound **IV** was the primary degradant detected in actual expired stability lots of Albalon-A using either HPLC method 1 or 2. Since the "real-time" degradation product was now identified, it was concluded that additional physiochemical stressing (pH extremes, light, etc.) of product was not necessary to prove assay specificity. Stability indication studies consisted of analyzing an expired Albalon-A stability lot which was spiked with **III-V**. Samples were analyzed using HPLC method 2 with the addition of a diode array detector for spectral acquisition of all analytes. Antazoline, naphazoline, and **IV** were all resolved from one another, and each demonstrated spectral homogeneity throughout their bandwidths, which demonstrated that the method was stability-indicating.

Standard curves for antazoline (100–1000 μg/mL) and naphazoline (10–100 μg/mL) both yielded linear plots with correlation coefficients (r) > 0.9999. Using synthesized **IV** as a calibration standard, degradant **IV** was determined to be present in the expired Albalon-A stability lot at 20 μg/mL (0.002% w/v). The **IV** standard curve was therefore generated from 5–40 μg/mL, which yielded a linear response with r = 0.9997. The detection limit for **IV** was determined to be 2 μg/mL using 30 μL injections (after sample dilution).

Accuracy studies (n = 6 replicates each), using placebo formulation as the spiking medium, were conducted for antazoline and naphazoline at their label claim; 0.5% and 0.05% w/v, respectively. Accuracy for **IV** was conducted at the 20 μg/mL concentration found in the expired stability sample. Table 1 lists the recovery values obtained. These data indicate that the assay is reproducible between days and

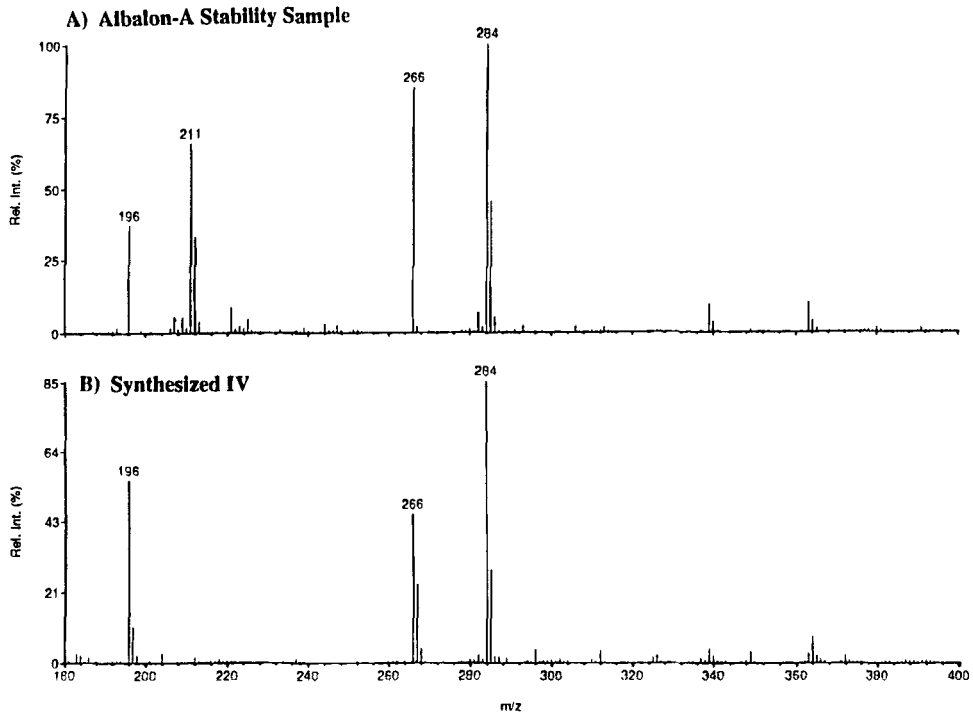

Figure 6—HPLC–MS Spectra obtained for compound **IV** detected in the analysis of (a) ophthalmic solution stability sample and (b) synthetic **IV** standard.

analysts to within ±1% for the active drugs and within ±4% for **IV**, which is considered suitably rugged, precise, and accurate for worldwide QA/QC stability applications.

Conclusion

N-[(*N*-Benzylanilino)acetyl]ethylenediamine (**IV**) was conclusively identified as the major degradation product found in expired lots of Albalon-A ophthalmic solution. Since **IV** was also found in antazoline phosphate raw material, at a concentration much lower than that detected in product, this degradation product must be formed via a hydrolysis pathway in the formulation after manufacture. The hydrolysis product **V** was not detected and antazoline could not be hydrolyzed (using the conditions described) past **IV**, indicating that the hydrolysis byproduct ethylenediamine is not expected to be present in this product, even after drug degradation has occurred. Using synthesized degradants and expired Albalon-A product, HPLC method 2 has been shown to be stability-indicating and specific for antazoline and naphazoline. In addition, the method described has been shown to be sufficiently rugged, accurate, and precise for stability testing.

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