BioProcessing JOURNAL
TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Investigation of Light Isomerization of Tropolone Using Liquid Chromatography and Orbitrap Mass Spectrometry

By ROBERT J. DUFF and JOHN L. SNYDER

Introduction

This investigation into the light isomerization of tropolone was initiated after a method for determining residual tropolone in the bulk drug substance (BDS) of a therapeutic monoclonal antibody was developed and validated at our laboratory. This method, developed at a client's request, required a detection limit of 50 parts per billion (ppb) in the BDS to meet their regulatory requirements. The method was developed using liquid chromatography and tandem mass spectrometry (LC/MS/MS) because of the selectivity and sensitivity of the technique when using selective reaction monitoring (SRM). During development, however, it was discovered that only after creating a photoisomer of tropolone could successful separation, detection, and quantitation of tropolone be achieved. During validation, the method showed good performance. Linearity was demonstrated over the range of 10–100 ng/mL. The limit of detection was established at 8 ng/mL, based on a peak to peak signal to noise greater than three. Accuracy was demonstrated by performing spiking studies in triplicate on the BDS sample over the concentration range of 20–200 ng/mL. Recoveries ranged from 86–94% and all relative standard deviations were less than 15%. However, uncertainty remained as to the origin of the peak and if that peak was truly a photoisomer of tropolone. Because of the low concentration of the peak, repeated efforts to isolate it and obtain nuclear magnetic resonance (NMR) spectra were unsuccessful. In this paper, we discuss the investigation that was conducted using Orbitrap mass spectrometry to confirm that the peak monitored by the LC/MS/MS method was indeed a photoisomer of tropolone, and also to identify the specific photoisomer.

Tropolone is composed of a seven-carbon ring with three double-bonds, therefore the molecule exhibits both aromatic and allylic properties (Figure 1). Two adjacent oxygen atoms are attached to the ring, causing the molecules to be in rapid equilibrium between two tautomers.[1] Tropolone, because it chelates with metal ions, can be used to aid in the uptake of iron and increase cell growth during the fermentation step in the manufacture of genetically engineered monoclonal antibodies. In fact, tropolone and ferric ammonium citrate, in conjunction with recombinant proteins such as human albumin and human insulin, can be used to help replace animal-source proteins not commonly found in serum-free media used in NS0 cell culture.[2]

Because it was necessary to demonstrate clearance of tropolone during post-culture purification, a method was developed using LC/MS/MS to detect tropolone at low ppb concentrations in the final bulk drug substance of a therapeutic monoclonal antibody. During development of this method, a satisfactory chromatographic separation of tropolone at ppb concentrations could not be obtained despite a myriad of approaches ranging from hydrophilic interaction chromatography to reversed-phase chromatography and the use of ion-pairing agents. In researching this enigma, it was found that Day and Ledlie[3] published a study on the photochemistry of α-tropolone in water, methanol, and hexane. The authors reported that tropolone formed a series of photoisomers when exposed to light. In their paper, they speculated on the number of pathways and photoisomers that could potentially form. Based on their work and a similar paper by Chapman and Pasto,[4] the method development strategy was changed. Tropolone was exposed to light and a photoisomer more amenable to liquid chromatography was monitored. This approach led to the successful development and validation of the method described in this paper.

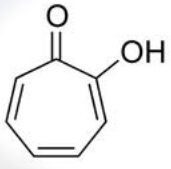


FIGURE 1. Chemical structure of tropolone. (*Courtesy of Wikipedia.*)

In this method, tropolone is converted to a photoisomer by exposing standard and sample solutions to light in a light chamber. The intensity and time of light exposure was controlled. Efforts to isolate the photoisomer peak and confirm its identity by proton and carbon NMR were unsuccessful. Carbon and proton NMR spectra were inconclusive. The crux of the problem was in isolating the peak of interest at a sufficient concentration to obtain NMR spectra. The peak that is monitored is formed after light exposure (765 W/m^2) for two hours. The formation of this peak at 5.7 min was shown in a time study during method development and is shown in Figure 2. Isolation of the photoisomer (peak 5.7 min) and the collection of a sufficient sample quantity for NMR analysis was extremely difficult because the reaction could not be replicated at higher concentrations. The chromatograms shown in Figure 2 were generated using SRM of the parent ion at a mass-to-charge ratio (m/z) of 123 and a daughter ion at m/z 77 of a tropolone standard in water at a concentration of 50 ppb. When higher concentrations of tropolone (*ca.* >10 ppm) are exposed to light, the peak at 3.6 min is produced but the reaction does not proceed to produce the peak at 5.7 min as shown earlier. SRM is exceptionally selective and did not detect other components with a different molecular weight or fragmentation pattern in the chromatogram. Interferences are rare but more probable at low molecular weight. Because of the failure to obtain quality NMR spectra of the peak at 3.6 min and the peak at 5.7 min, an investigation was conducted using liquid chromatography and an Orbitrap mass spectrometer. The Orbitrap can acquire scan data, accurate mass data, and scan dependent daughter ion scans in MS/MS mode (MS2), and also perform SRM, although the sensitivity is not as good as what can be seen with the triple quadrupole mass spectrometer.

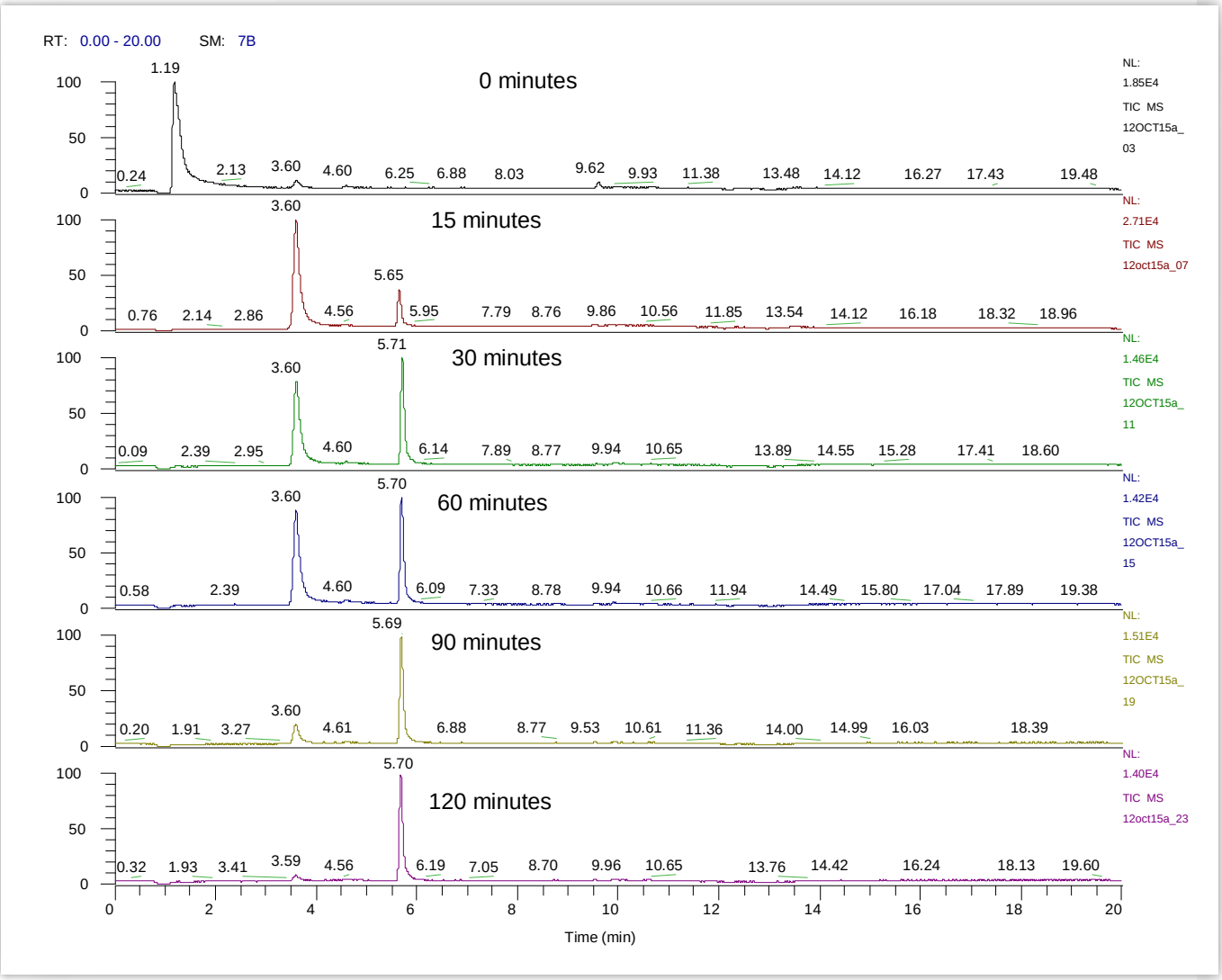


FIGURE 2. Example SRM chromatograms of a tropolone standard (50 ng/mL) with increasing time exposure to light. **Top to bottom:** 0 to 120 minutes.

Materials and Methods

The tropolone standard used in these experiments was made in water at: (a) 1 mg/mL; (b) 100 µg/mL; (c) 10 µg/mL; and (d) 1 µg/mL. Approximately 10 mL of each standard was exposed in the light chamber set at 765 W/m^2. These standards were contained in clear glass 25 mL vials and secured horizontally in the light chamber to maximize exposure. Subsamples were taken from the vials at time zero and at 30-minute intervals over a period of two hours. These subsamples were injected into an ultra performance liquid chromatograph (UPLC) instrument (Waters ACQUITY UPLC H-Class) interfaced to a Thermo Scientific™ LTQ Orbitrap Mass Spectrometer. The Orbitrap system is a very high resolution mass spectrometer incorporating Fourier transform ion cyclotron resonance (FTMS). Instrument conditions are summarized in Table 1. The UPLC system was equipped with a UV detector connected in series prior to the mass spectrometer. The Orbitrap mass spectrometer was calibrated prior to data acquisition. All data were acquired using only the Orbitrap FTMS to insure mass accuracy.

NMR spectra were obtained from Spectral Data Services, Inc. Tropolone (Fluka™ Lot #BHBH0032V, Sigma-Aldrich Co.) was dissolved in deuterated water (D_2O). The proton NMR spectrum was obtained on a 400 MHz instrument and the sample was spun at 20 Hz at 25°C. The sweep width was 7 kHz and 40 transients were performed.

TABLE 1. Instrument conditions on the UPLC and Orbitrap mass spectrometer.

Parameter	Condition	
LC/MS model	UPLC and mass spectrometer	
Injection volume	10 µL	
Mobile phase A	0.1% v/v formic acid in water	
Mobile phase B	Methanol with 0.1% v/v formic acid	
Temperatures	30°C (column); 50°C (autosampler tray)	
Column	Phenomenex® Synergi™ Polar RP, 2.5 µm, 2.0 × 100 mm	
Flow	200 µL/min	
Gradient	Time (min)	% B
	0.0 10.0 15.0 Re-equilibrate to start	2.0 90.0 90.0
UV detector	254 nm	
Ionization	Atmospheric pressure electrospray ionization	
Polarity	Positive	
Tune file	Tropolone_01	
Capillary temperature	350°C	
Sheath gas pressure	60 (arbitrary units)	
Auxiliary gas pressure	10 (arbitrary units)	
FTMS	Scan m/z 50–500	
MS/MS	FTMS scan-dependent data after collision induced dissociation (CID) (top 2)	
FTMS SRM	m/z 123 → m/z (76–78)	

Results and Discussion

Four major peaks were observed during the light study. The native tropolone peak was not observed when injected at concentrations of approximately 10 µg/mL or less. This is illustrated by Figures 3 and 4.

The peak at 12.47 min in the base peak chromatogram was present in the blank (m/z 338) and was not related to tropolone. When the concentration of tropolone was increased to 100 ppm for injection, with no light exposure, a peak at approximately 6.4 min was observed (as shown in Figure 4).

The native form of tropolone was possibly binding to the column at low concentrations. This was observed during method development and could not be explained. The native tropolone will be referred to as "peak 1" in the

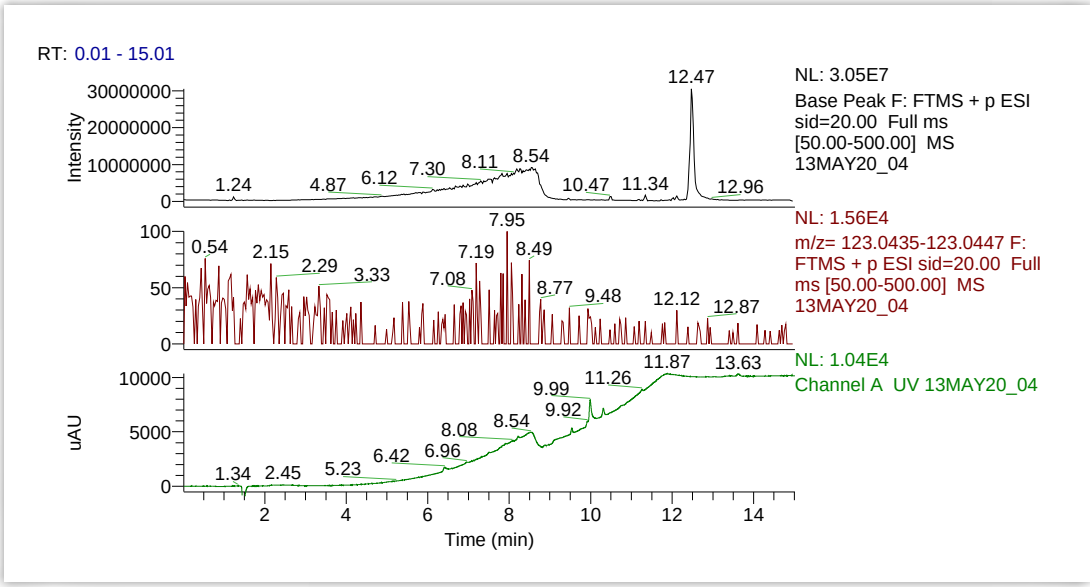


FIGURE 3. Example chromatograms of tropolone at 10 ppm in water and no light exposure. **Top:** base peak chromatogram; **Middle:** extracted ion chromatogram m/z 123; **Bottom:** UV 254 nm.

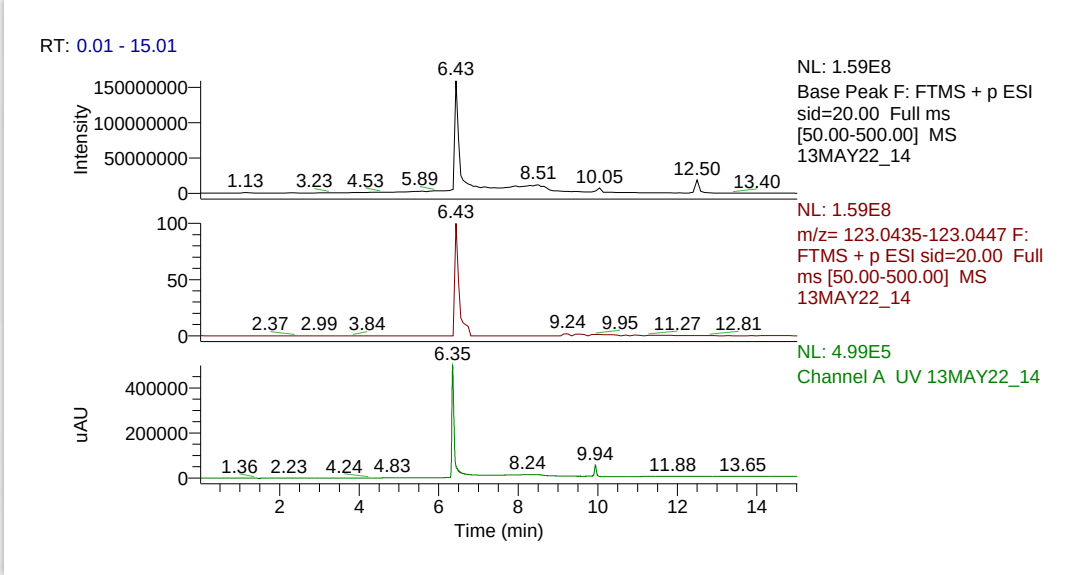


FIGURE 4. Example chromatograms of tropolone at 100 ppm in water and no light exposure. **Top:** base peak chromatogram; **Middle:** extracted ion chromatogram m/z 123; **Bottom:** UV 254 nm.

following discussion. Figure 5 presents the MS spectrum of tropolone showing the molecular $(M+H)^+$ ion at m/z 123, as well as the elemental composition that best fits this molecular mass.

After exposure to light for 30 min, a major peak at 3.6 min began to appear ("peak 2"), as shown in Figure 6. This solution was treated at approximately 1 mg/mL, diluted 100-fold to a final concentration of 10 ppm for analysis.

The compound represented by this peak (3.6 min) was subcontracted for proton NMR analysis, returning inconclusive results. The proton NMR spectrum of pure tropolone is shown in Figure 7. The spectrum shows the

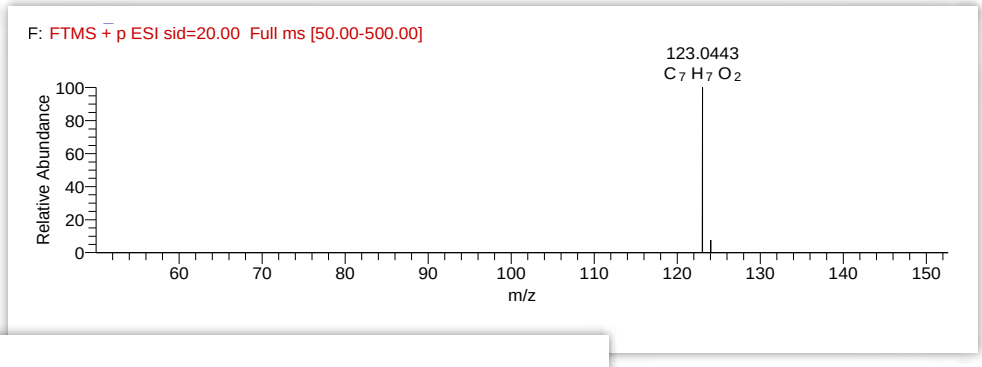


FIGURE 5. Spectrum example of tropolone acquired on the Orbitrap MS with no light exposure.

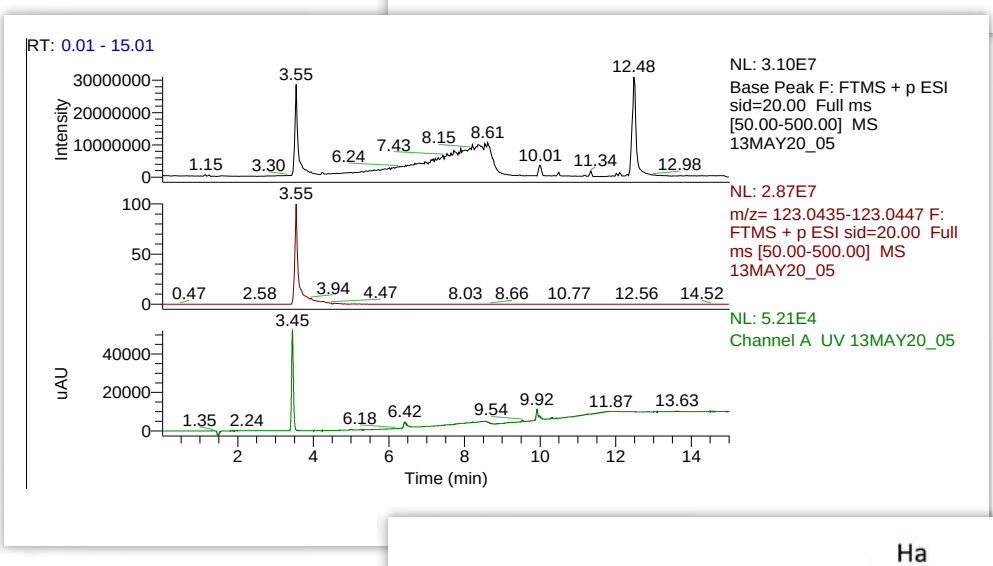


FIGURE 6. Example chromatograms of tropolone (1 mg/mL) exposed for 30 min, diluted to 10 ppm in water. **Top:** base peak chromatogram; **Middle:** extracted ion chromatogram m/z 123; **Bottom:** UV 254 nm.

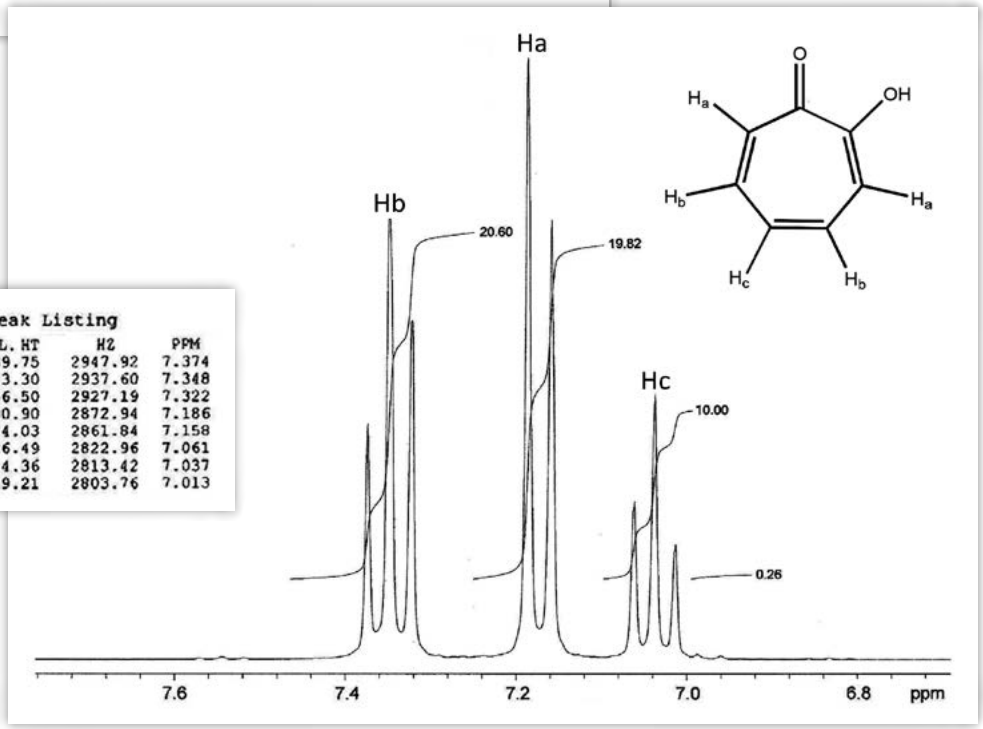


FIGURE 7. NMR (400 Hz) spectrum of native tropolone showing three distinct patterns.

Interpolated Peak Listing

PEAK	POINT	HEIGHT	REL. HT	HZ	PPM
1	15771	7911K	39.75	2947.92	7.374
2	15819	14587K	73.30	2937.60	7.348
3	15868	11245K	56.50	2927.19	7.322
4	16122	20081K	100.90	2872.94	7.186
5	16174	14733K	74.03	2861.84	7.158
6	16356	5271K	26.49	2822.96	7.061
7	16401	8829K	44.36	2813.42	7.037
8	16446	3824K	19.21	2803.76	7.013

three distinct regions related to the proton environments in the molecule as expected for a symmetrical, tautomeric compound (Figure 8).

The complexities of the light-treated NMR stem from the limited amount of material used for each experiment. NMR studies usually require milligram quantities of a pure compound for a confident structural identification to be made. Moreover, the chromatographic separation was not sufficient, nor would it ever be sufficient to separate the proposed diastereomeric products. With that stated, MS has the potential to discern these diastereomeric compounds at much lower concentrations. When a less concentrated solution of tropolone (10 ppm) was exposed to light for 30 min, two additional peaks were observed, as shown in Figure 9. The peak at 4.3 min ("peak 3") was not observed using SRM (m/z 123 → m/z 77) during method development. Further discussion about peak 3 is provided later in this article. The peak observed at 5.3 min ("peak 4") was determined to be the peak that was monitored by the LC/MS/MS method. Figure 10 shows chromatograms of a 100 ppm standard of tropolone exposed for two hours in the light chamber.

Peak 3 (at 4.3 min), is not detected by SRM setup for tropolone

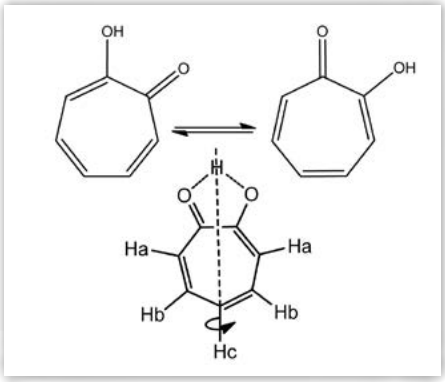
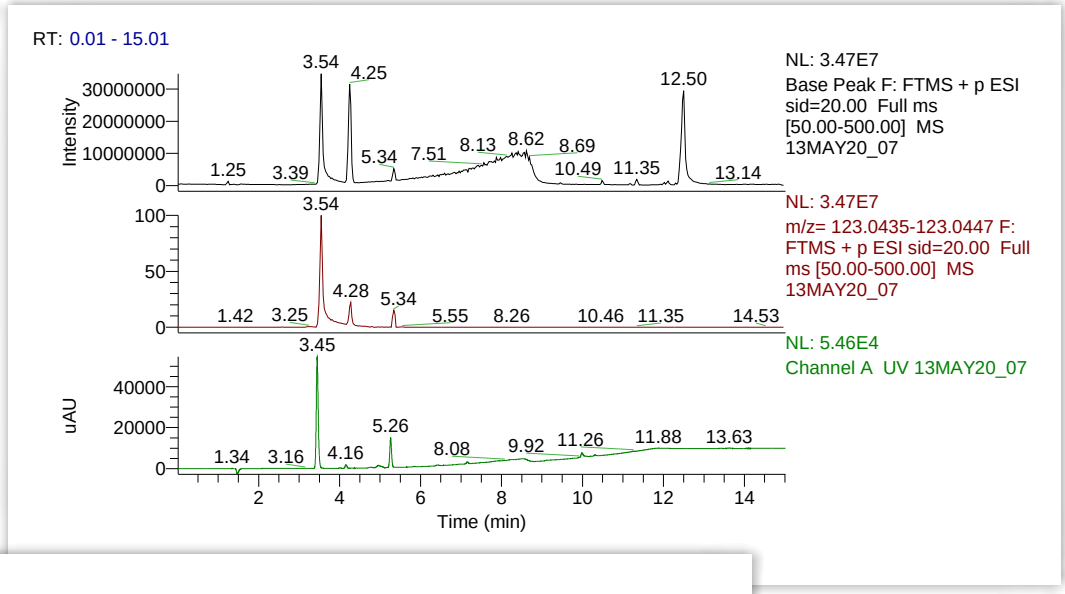


FIGURE 8. Tautomeric forms of tropolone and inset plane of symmetry, as related to proton chemical shift equivalency.



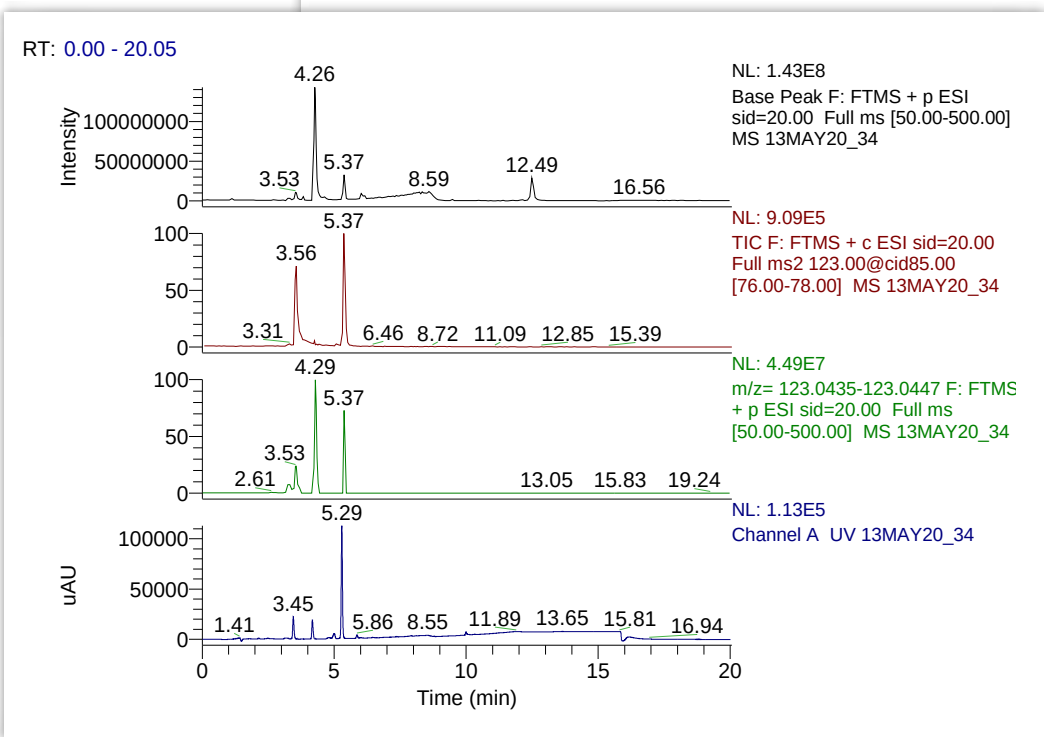


FIGURE 9 (above). Example chromatograms of tropolone (10 ppm) exposed for 30 min and injected directly; **Top:** base peak chromatogram; **Middle:** extracted ion chromatogram m/z 123; **Bottom:** UV 254 nm.

FIGURE 10 (left). Example chromatograms of tropolone at 100 ppm in water and 120 minutes exposure; **Top:** base peak chromatogram; **Middle Top:** SRM m/z 123 → m/z 77; **Middle Bottom:** extracted ion chromatogram m/z 123; **Bottom:** UV 54 nm.

because its molecular weight (*ca.* 154 Da) does not match that of tropolone. Therefore peak 3 is not an isomer, although it is related to tropolone. MS and MS2 examples of peaks 3 and 4 are shown in Figures 11 and 12. The spectrum of peak 3 (Figure 9, top frame) has a molecular ion interpreted at m/z 155. The MS spectrum of peak 4 (Figure 11, bottom frame), monitored in this method, has a molecular ion at m/z 123 and exactly matches the mass of native tropolone, making this peak an isomer. Normally the molecular ion is the base peak (largest) in an electrospray spectrum, but peak 3 showed a smaller molecular ion at m/z 155 and larger ion at m/z 141 (minus CH_2), which is the base peak in this spectrum.

Daughter ion scans (or MS2 spectra) for each of the four peaks are presented in Figures 12-13 (below) and Figures 14-15 (on the following page). Each of these spectra was generated using CID and acquired on the FTMS for accurate mass measurement. The elemental composition of the fragment ions are presented above the ions. The daughter ion scan of peak 3 does not show a significant daughter ion at m/z 77 and, therefore, this peak was not monitored by triple quadrupole methods. The peak 4 MS2 spectrum is shown in Figure 15.

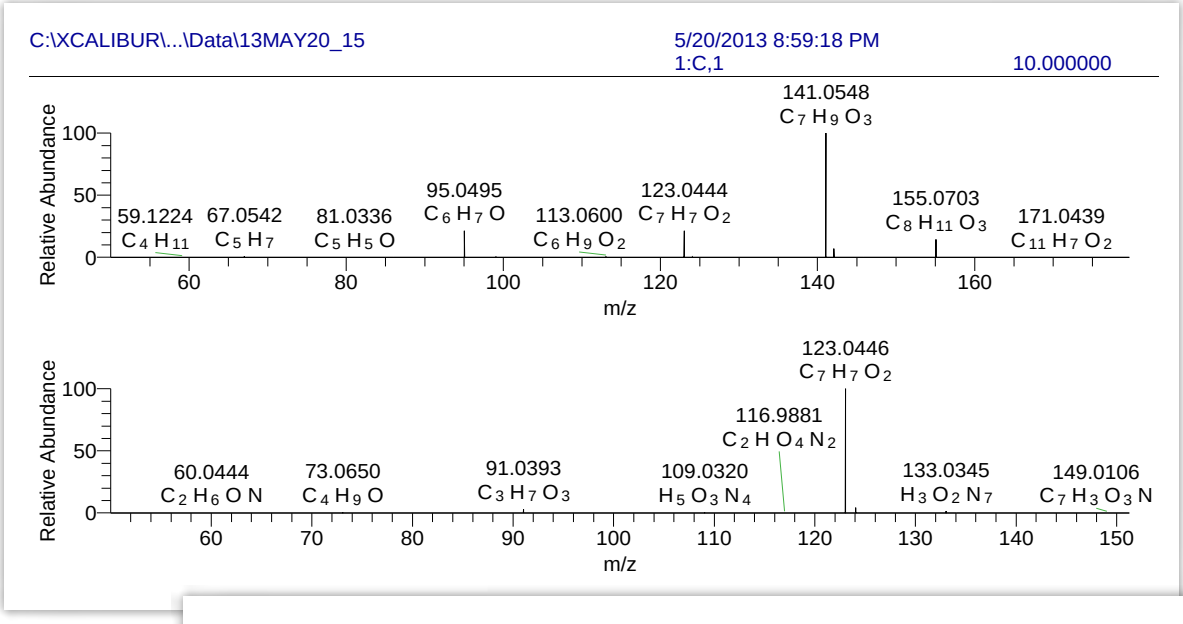


FIGURE 11. MS spectrum. **Top:** peak 3; **Bottom:** peak 4.

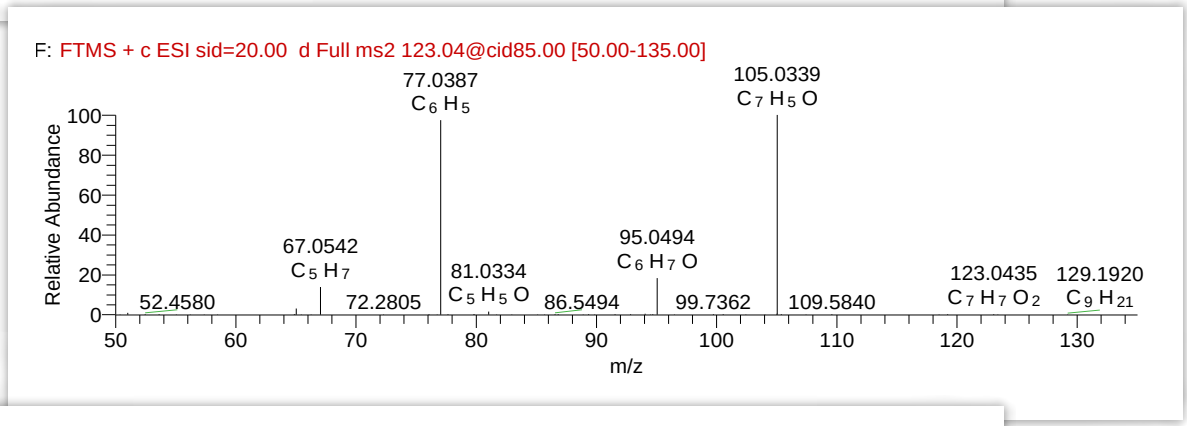


FIGURE 12. MS/MS spectrum of peak 1 (native tropolone); MS2 scan of m/z 123.

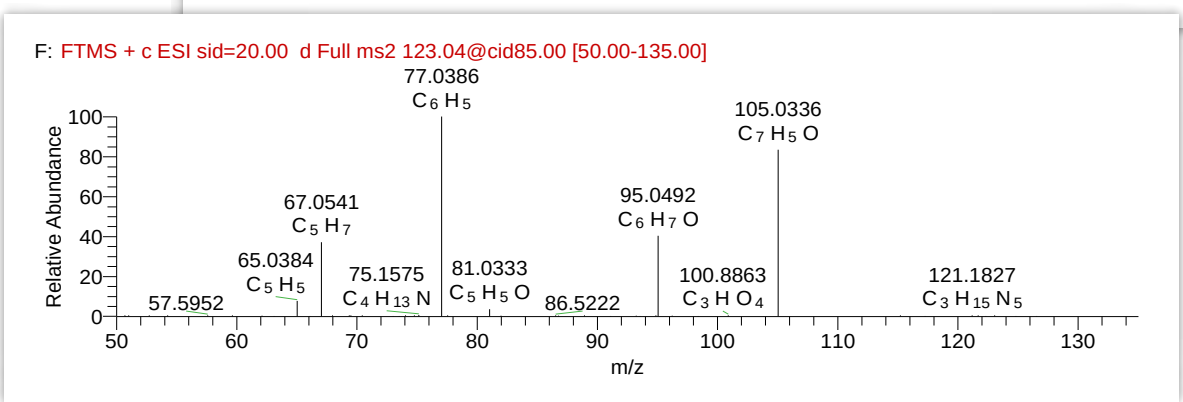


FIGURE 13. MS/MS spectrum of peak 2; MS2 scan of m/z 123.

Conclusion

Four major peaks were observed and identified during this light exposure study of tropolone. Peak 4 was identified as the photoisomer of tropolone monitored by the LC/MS/MS method developed in our laboratory. A summary of these peaks is presented in Table 2. Structures related to tropolone are presented in Figure 16. Several of

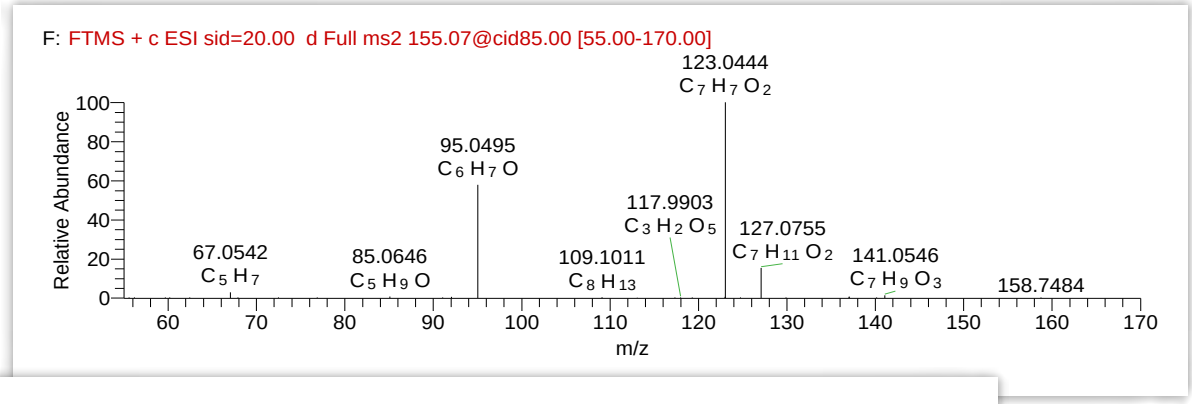


FIGURE 14. MS/MS spectrum of peak 3 MS2 scan of m/z 151.

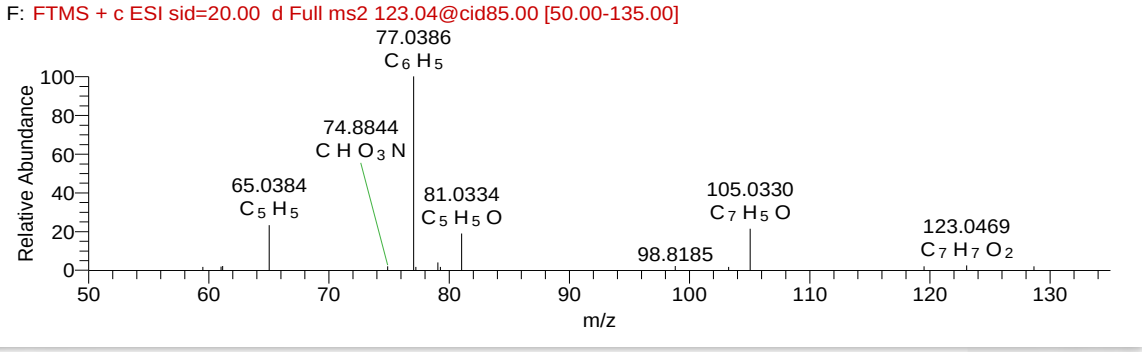


FIGURE 15. MS/MS spectrum of peak 4 MS2 scan of m/z 123.

TABLE 2. Tropolone-related peaks.

Peak #	Retention Time (minutes)	Chemical Formulation*	Mass (Da)	Comment
1	6.4	$C_7H_6O_2$	122.0368	Native tropolone. Peak only observed at 100 ppm and not at lower concentrations ≤ 10 ppm.
2	3.6	$C_7H_6O_2$	122.0368	First peak formed during light exposure. Tropolone isomer formed at elevated concentrations (1 mg/mL).
3	4.3	$C_8H_{10}O_2$	154.1630	Methyl ester of tropolone. Not observed by LC/MS/MS method.
4	5.3	$C_7H_6O_2$	122.0368	Monitored by the LC/MS/MS method. This isomer does not readily form at higher concentrations (1 mg/mL).

*Neutral species and monoisotopic mass presented

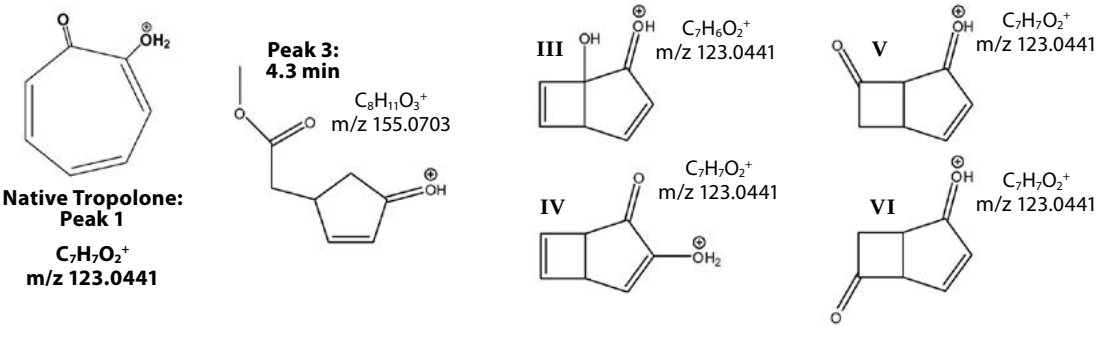


FIGURE 16. Possible photoisomers of tropolone[3] presented as molecular ions.

A robust & flexible universal bioreactor control system from 1L to 2000L. Powered by Finesse TruBio® software.

G3Lab

G3Pro

G3Flex

Universal Bioreactor Control Platform

The perfect control for bioprocess management.

Smart Controllers

Unify upstream processing with one common user platform

Scale up and down easily between R&D and cGMP

Interchange vessels of any size or from any vendor

Simplify with our easy-to-use interface and data processing

Leverage the power of a DeltaV® or Wintel control system

Increase uptime and reliability with our full line of services

Make your process excellent.

USA Toll Free +1 800 598 9515 | Europe +41 81 641 2000 | Asia +65 8322 8128 | Finesse.com

Finesse

these structures are isomers[3] and all are presented as the positive molecular ion.

The native tropolone (peak 1, no light exposure) was only detected at a concentration of approximately 100 ppm. No peaks were visible in the unexposed tropolone standard when injected at 10 ppm. The peak monitored by the quantitative method using SRM (peak 4) was confirmed to be an isomer of tropolone because the exact mass of this peak matched that of native tropolone (123.0441 Da, $M+H^+$ ion) and the MS2 spectra contained similar fragment ions. The peak that formed most readily during light exposure (peak 2) was also determined to be an isomer of tropolone based on its exact mass and MS2 spectrum. Peak 3, not detected by the SRM method (m/z 123 → m/z 77), was discovered to be present and related to tropolone but not an isomer. This peak had a molecular ion at m/z 155 and was a methyl ester.

Identification of exact isomers from the fragmentation patterns was not accomplished during this study because of the similarity of the fragmentation patterns in the MS2 spectra. The MS2 spectra of the peaks showed similar fragment ions, and for some of the isomers, only slight differences in relative abundance. The symmetry and tauterism exhibited by the tropolone added to this complexity. In a future study, it may be beneficial to obtain tropolone with an alkyl group substituted for either the Ha or Hb hydrogen atom (see Figure 8). The compound β-thujaplicin (2-hydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one), which is commercially available, has an isopropyl group attached at this position and could facilitate unambiguous interpretation and discernment of the MS2 spectra. The presence of this isopropyl group would inhibit the tauterism exhibited by the tropolone molecule.

References

[1] Weiler L. 13C-nuclear magnetic resonance spectrum of tropolone. *Canadian J Chem*, 1971; 50: 1975-1977. http://dx.doi.org/10.1139/v72-316.

[2] Zhang J, Robinson D. Development of animal-free, protein-free, and chemically-defined media for NS0 cell culture. *Cytotechnol*, 2005; 48: 59-74. http://dx.doi.org/10.1007/s10616-005-3563-z. PMID 190030323A.

[3] Day C, Ledlie MA. The photoisomerisation of α-tropolone. *J Chem Soc D, Chemical Communications*, 1970; 19: 1265-1266. http://dx.doi.org/10.1039/c2970001265b.

[4] Chapman OL, Pasto DJ. Photochemical transformations of simple troponoid systems. *J Am Chem Soc*, 1960; 82 (14): 3642-3648. http://dx.doi.org/10.1021/ja01499a044.

About the Authors

Robert J. Duff, PhD (RDuff@lancasterlabs.com) is Manager, Biopharmaceutical Services; and
John L. Snyder, PhD (JSnyder@lancasterlabs.com) is Principal Chemist, Biopharmaceutical Services.

eurofins Lancaster Laboratories
2430 New Holland Pike, Lancaster, Pennsylvania USA 17601; Phone: 717-656-2300