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The use of LC/MS, GC/MS, and LC/NMR hyphenated techniques to identify a drug degradation product in pharmaceutical development

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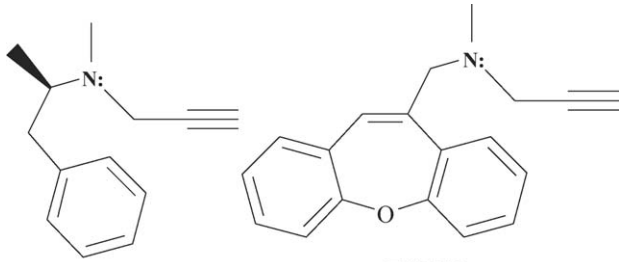
Abstract

Understanding drug degradation in the formulated product is critical in pharmaceutical development as it has significant impacts on drug efficacy, safety profile and storage conditions. As a result, identification of degradation compounds has taken an important role in the drug development process. In this study, various hyphenated analytical techniques, such as liquid chromatography mass spectrometry (LC/MS), gas chromatography mass spectrometry (GC/MS), and liquid chromatography nuclear magnetic resonance with a solid phase extraction interface (LC/SPE/NMR), have been applied to the identification of a drug degradation product which grew over time in the stability study of the drug product. The target unknown is less polar and more unsaturated than the drug substance based upon reverse phase HPLC relative retention time and UV spectra. It is not ionizable by electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) in either a positive or a negative mode. The unknown was isolated by an HPLC fraction collector and enriched by solid phase extraction. GC/MS with chemical ionization (CI) was employed to determine the molecular weight of this compound. Its fragmentation pattern was determined by CI-MS/MS using an ion trap mass spectrometer. The isolated material was also analyzed by LC/SPE/NMR, from which the structure of this compound was further characterized. The study utilizes a combination of various hyphenated analytical techniques to obtain complimentary information for structure elucidation of the unknown. The combination approach is critical for unambiguous impurity structure elucidation in drug degradation studies of pharmaceutical drug products.

Keywords: LC/MS; GC/MS/MS; LC/SPE/NMR; Drug degradation

1. Introduction

TCH346 (dibenzo[b,f]oxepin-10-ylmethyl-methyl-prop-2-ynyl-amine hydrogen maleate) is a novel chemical entity which is being investigated for the treatment of neurodegenerative disorders, such as Parkinson's disease and amyotrophic lateral sclerosis. TCH346 is a low molecular weight compound which belongs to a family of amino-methyl-dibenzoxepines, structurally and functionally related to *R*-(-)-deprenyl (Selegiline, see structure below). *R*-(-)-Deprenyl has been used for a long time in the treatment of Parkinson's disease due to its neuroprotective effects which has been demonstrated in vivo [1–4] and in vitro[5–8]. The studies [9–11] indicate that TCH346 is two orders of magnitude more potent than *R*-(-)-deprenyl. It shows excellent brain penetration and is expected to be capable of slowing or halting the progression of Parkinson's disease.



R-(-)-Deprenyl TCH346

Hyphenated analytical techniques such as LC/DAD-UV, LC/MS, GC/MS and LC/NMR, in which a chromatographic separation is coupled online with one or more information-rich detectors, have quickly become indispensable tools for low level impurity identification or confirmation. All of these techniques have complementary selectivity, often requiring analysis by all

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of them to completely define an unknown molecular structure. While the LC/DAD and LC/MS instruments are well-developed laboratory tools now widely used in the pharmaceutical industry, LC/NMR has only recently overcome the sensitivity limitations historically impeding its widespread application in the pharmaceutical industry [12,13]. The availability of cryogenically cooled low-volume probes [14,15] and C/N data from indirect proton detection using polarization transfer techniques [16] has radically increased the amount of information available from a small amount of analyte in the NMR coils. An SPE trapping interface between the LC column and the NMR [17–20] now allows on-line concentration and purification of very low-level minor components into a low-volume flow cell for high quality NMR spectral data.

TCH346 is a propargylamine compound with a pK_a at 6.3. TCH346 drug substance is chemically stable, however, a number of degradation products were observed in the forced degradation at high pH of a TCH346 tablet formulation (unpublished results). One unknown degradation product observed during the storage conditions (25 °C/60% RH and 40 °C/75% RH) of the TCH346 drug product grew over time. Identification of this unknown is particularly challenged due to its poor ionization properties and difficult separation from other basic drug impurities in HPLC. Stress studies indicate that this degradation product can be produced in the basic condition under Xenon light, indicative of photo-catalytic oxidation of the TCH346 drug substance. In this study, extensive studies were conducted to characterize this degradation product using various hyphenated analytical techniques, such as LC/MS with ESI and APCI, peak isolation by LC fraction collection and enrichment by solid phase extraction (SPE), and GC/MS/MS with chemical ionization. The molecular structure was identified and confirmed by LC/NMR with a solid phase extraction.

2. Experimental

2.1. Liquid chromatography/mass spectrometry

Mass spectrometry. All LC/MS mass spectral data were collected using an LCQ^{Deca} Ion Trap Mass Spectrometer (ThermoElectron, San Jose, CA, USA) equipped with both ESI and APCI sources. In order to ionize the target compound, both positive (+) and negative ESI (−) modes as well as positive APCI modes were investigated. The mass range acquired was from 215 to 1500 amu. ESI conditions: capillary temperature 350 °C, capillary voltage 3 V (positive mode) or −13 V (negative mode), sheath gas 80 (arbitrary units), auxiliary gas 10 (arbitrary units). APCI (+) conditions: capillary temperature 200 °C, vaporizer temperature 450 °C, capillary voltage 32 V, sheath gas 70 (arbitrary units), auxiliary gas 5 (arbitrary units).

Liquid chromatography. A 2690 Alliance HPLC system (Waters, Milford, MA, USA) was used in this study. The separation was achieved using a Waters YMC^{TM} ODS-AQ, 4.6 mm × 50 mm column with particle size of 3.0 μm and pore size of 120 Å (YMC, Inc. USA). Four HPLC mobile phase systems with different pH ranges were investigated to ionize the compound of interest under either a positive or a negative ionization mode. Mobile phase A for each of the four conditions: 0.1% trifluoroacetic acid (Aldrich, Milwaukee, Wisconsin, USA), 0.1% formic acid (Aldrich, Milwaukee, Wisconsin, USA), 20 mM ammonium acetate (Aldrich, Milwaukee, Wisconsin, USA) [pH 5.8], or 20 mM ammonium carbonate (Aldrich, Milwaukee, Wisconsin, USA) [pH 8.7]; in each case using acetonitrile (Fisher, Fair Lawn, New Jersey, USA) as the mobile phase B. The same HPLC gradient program was applied to all four cases: 1.5 ml/min flow rate; 0–2.4 min 10% B, 2.4–12.0 min from 10% B to 70% B, and held at 70% B for 2.5 min before returning to the initial conditions of 10% B; column temperature 25 °C; sample solvent: water–acetonitrile (60:40, v/v). A UV6000 LP PDA diode array detector (ThermoElectron, San Jose, CA, USA) was used to monitor the UV–vis signals at both 281 nm and 190–500 nm range.

2.2. Isolation and enrichment

The peak of interest was isolated and collected using a fraction collector (Waters Fraction Collector II interfaced with Waters 2695 HPLC system). In order to generate sufficient amount of the unknown for fraction collector, 10 mg/ml TCH346 solution in 0.1N NaOH was exposed to Xenon light (Heraeus SunTest, Phoeniz, AZ, USA) for 30 h with 2.4 million lux-h, then the stressed solutions was adjusted to pH5.0 by adding 0.1N HCl (Aldrich, Milwaukee, Wisconsin, USA). A Waters YMC^{TM} ODS-AQ, C18 m 4.6 mm × 50 mm column with particle size of 3.0 μm (YMC, Inc. USA) was used for isolation. The mobile phase A contained a mixture of sodium perchlorate (Aldrich, Milwaukee, Wisconsin, USA)–perchloric acid (Aldrich, Milwaukee, Wisconsin, USA)–water (1.22:0.5:1000, w/v/v) and the mobile phase B contained acetonitrile. The HPLC gradient program: 1.5 ml/min flow rate, 0–9 min from 15 to 46% B, 9–12 min from 46 to 70% B, held at 70% B for 2 min before returning to the initial condition. The injection volume was 200 μl. Over 300 injections were collected using a fraction collector. The collections were combined and placed into a vacuum drying oven (Baxter DP3-2), which was operated under vacuum at 50 °C to remove acetonitrile. The resulting aqueous portion then passed through the solid phase extraction cartridge (Sep-Pak Vac RC, 500 mg, C18), followed by adding 20 ml of water to wash the cartridge. About 15 ml of acetonitrile was then applied to the cartridge to elute the trapped compound. The elute was then dried in the same vacuum drying oven at 30 °C. The purity of the final isolated material was over 80%, as determined by HPLC.

2.3. Gas chromatography/mass spectrometry

The gas chromatograph was a TRACE GC-2000 (ThermoElectron, San Jose, CA, USA) equipped with both El and CI sources. The capillary column used was a J & W DB-5MS, 30 m × 0.25 mm i.d. × 0.25 μm film thickness (Agilent Technologies, Wilmington, DE, USA). The carrier gas was high purity helium (99.999%, Welding Supply Co., Inc., NJ, USA) under a constant column flow rate of 1 ml/min. A gas purifier

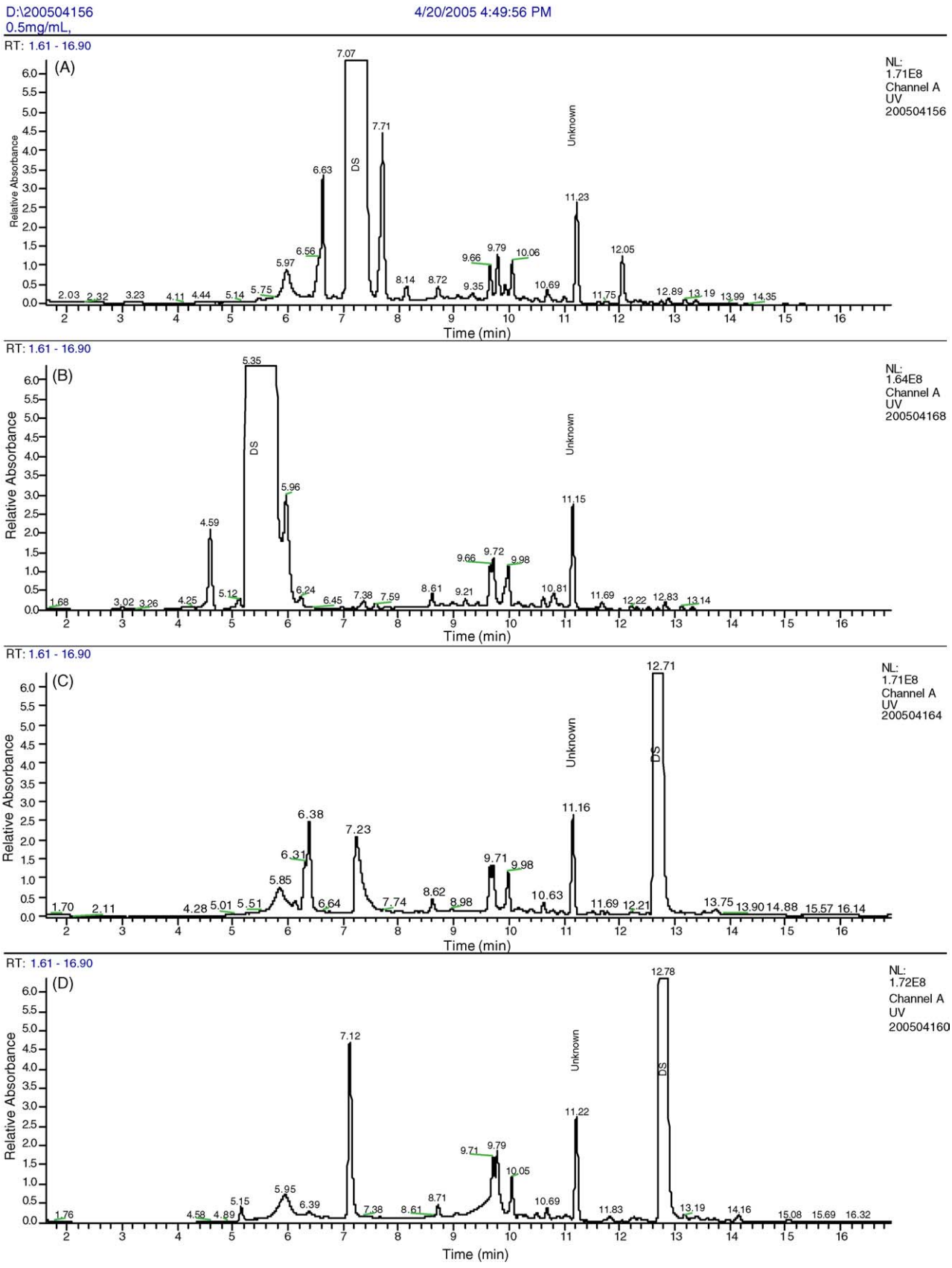


Fig. 1. HPLC chromatograms of the sample solution of TCH346 0.25 mg tablet stored at 40 °C/75% RH for 6 months. Mobile phase A: (A) 0.1% trifluoroacetic acid (TFA); (B) 0.1% formic acid (HCOOH); (C) 20 mM ammonium acetate (CH_3COONH_4), pH 5.8; and (D) 20 mM ammonium carbonate $(NH_4)_2CO_3$, pH 8.7. Mobile phase B: acetonitrile in each case. The same gradient condition was used for all four cases.

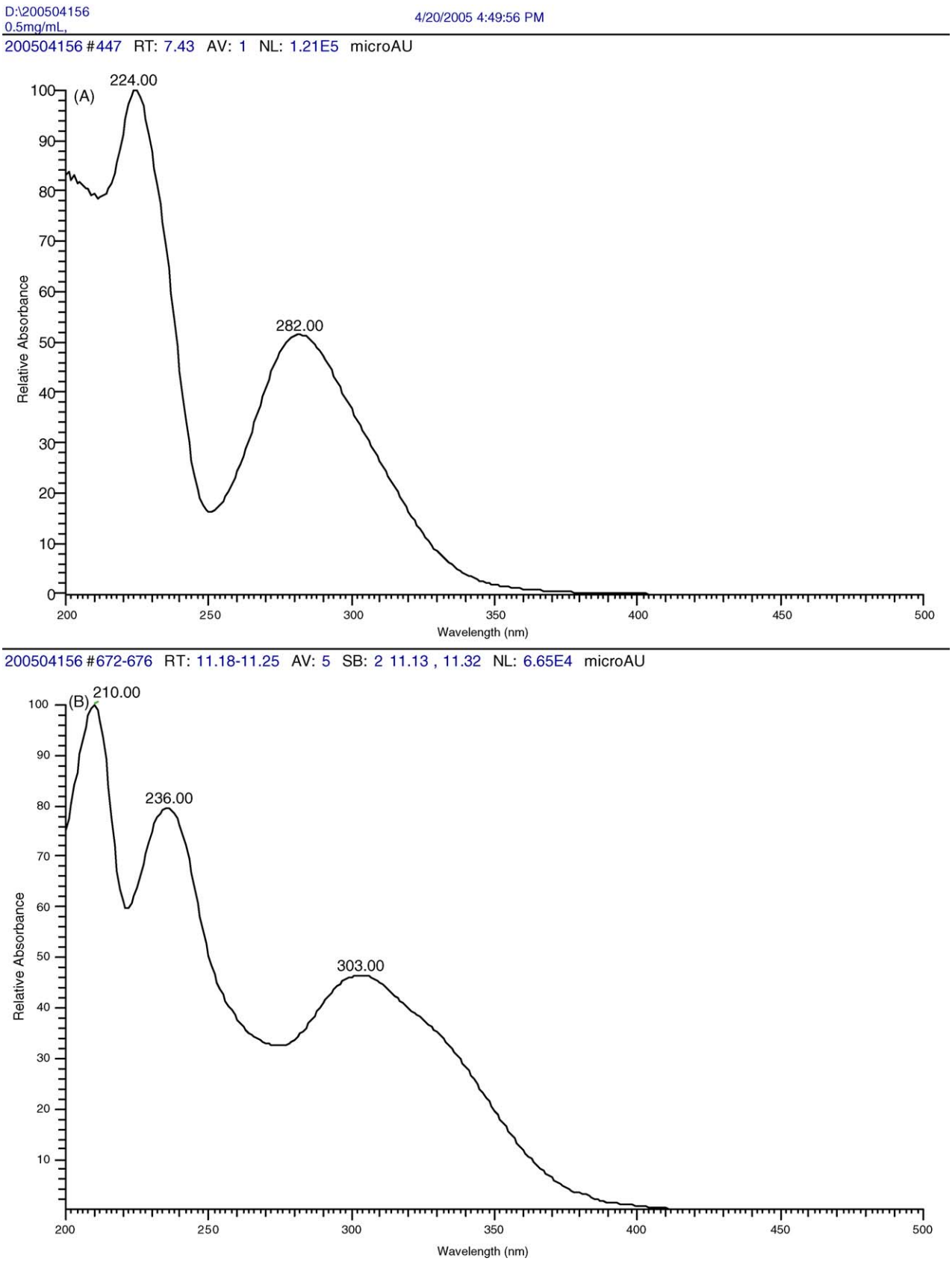


Fig. 2. UV–vis spectra of the TCH346 drug substance (A) and the unknown (B).

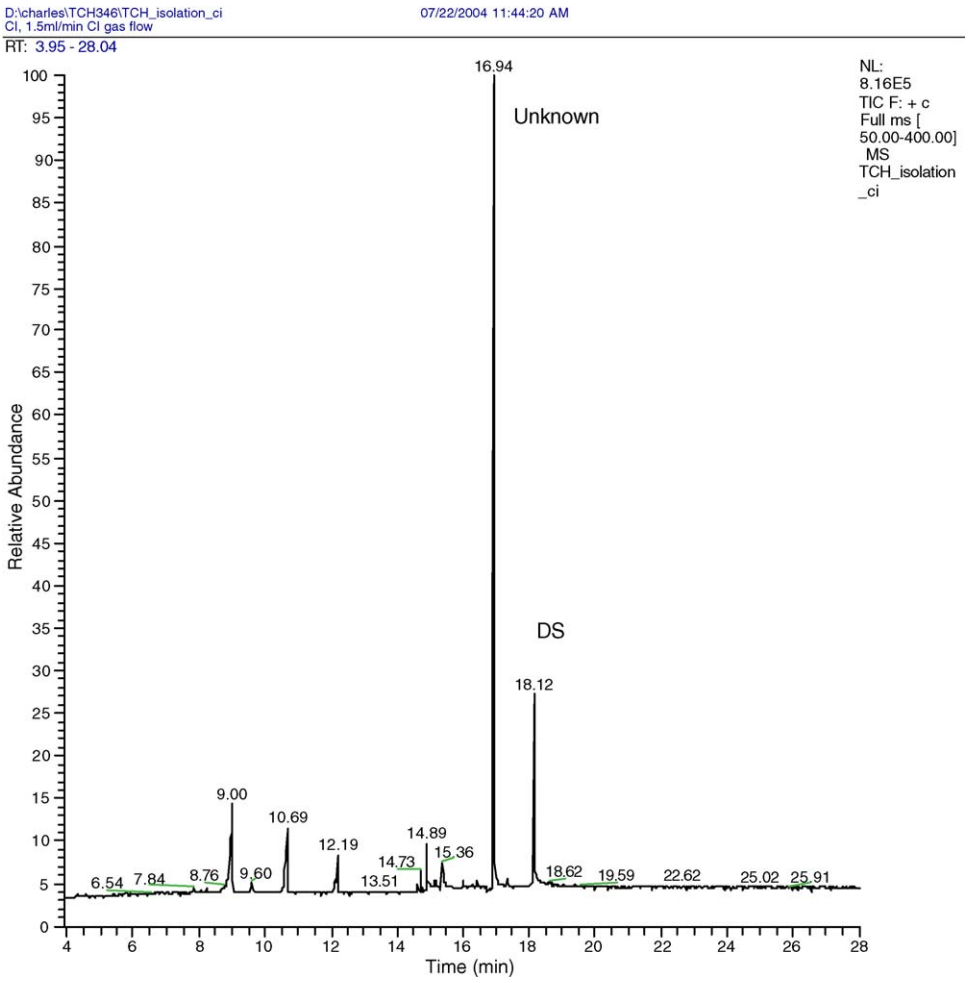


Fig. 3. GC/MS chromatogram of the isolated material.

(VICI, Fisher, PN 05730-2) and a moisture trap (VICI, Fisher, PN 05-730-9) were connected in series on the helium line to remove hydrocarbon impurities and trace water in the helium gas. The oven column temperature was initiated at 40 °C, held for 2 min, raised to 280 °C by 15 °C/min, and held at that temperature for 10 min. The injector temperature was 300 °C and the injection was operated in the splitless mode. The split vent was set at 20 ml/min and the septum purge was set at 1 ml/min. The GC/MS interface was set at 300 °C. The PolarisQ Ion Trap Mass Spectrometer (ThermoElectron, San Jose, CA, USA) was used to obtain GC/MS/MS spectra on the protonated molecular ion $[M+H]^+$ of this unknown with a collision energy of 1 V with a chemical ionization source installed in the mass spectrometer. The reagent gas was methane (Welding Supply Co., Inc, NJ, USA) at a flow rate of 1.5 ml/min. The mass spectral scans were carried out continuously from 50 to 400 amu during GC analyses with an ion source temperature at 220 °C.

2.4. LC/SPE/NMR

Approximately 200 μg of isolated impurity was dissolved in 200 μl acetonitrile for LC/SPE/NMR analysis. The analyte, now purified by a complementary LC separation, was recovered from the NMR flow cell and returned for MS confirmation.

The LC-UV-SPE-NMR measurements were carried out on a chromatographic separation system consisting of an Agilent 1100 G1313A autosampler, Agilent 1100 G1312A binary pump and Agilent 1100 G1315B DAD UV detector(Agilent Technologies, Wilmington, DE, USA). A single 200 μl injection was made by the autosampler into a reversed phase HPLC column. The peak of interest was automatically detected by UV absorbance at 290 nm for trapping by the Prospekt 2 solid phase extraction robot (Bruker BioSpin, Belarica, MA and Spark Holland, Plainsboro, NJ, USA) onto a Hysphere GP cartridge (2-mm i.d., 10 mm bed length, 10–12 μm particle size) after postcolumn addition of water using a Knauer K100 HPLC pump (Berlin, Germany). Prior to use, the cartridge was cleaned with acetonitrile and conditioned with water. The trapped analyte was dried with N_2 gas flow for 30 min, and eluted at 0.2 ml/min with $CDCl_3$ (“100%” grade, Cambridge Isotope Laboratories, Andover, MA) into the NMR cryo-flow cell for spectroscopic analysis.

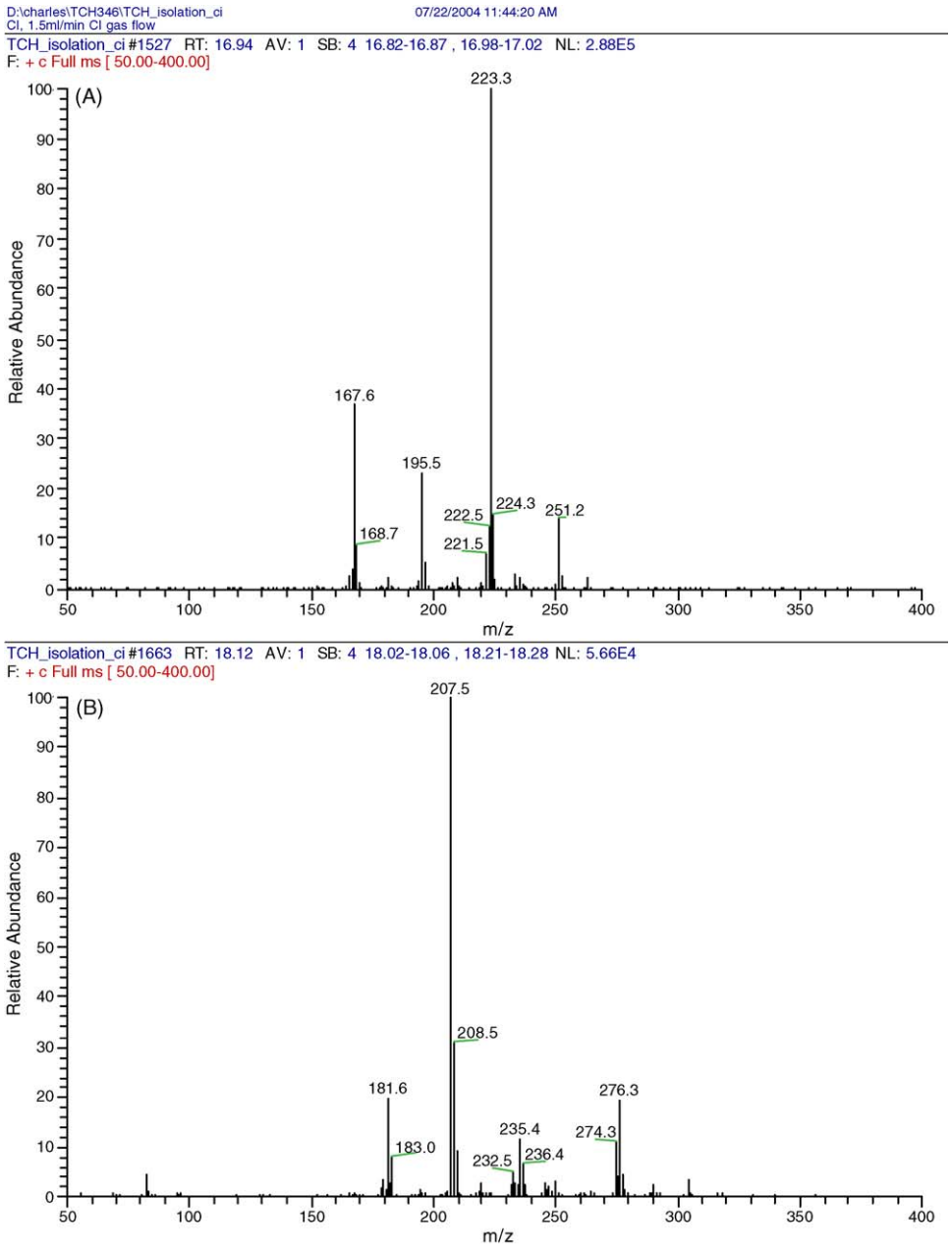


Fig. 4. Chemical impact (CI) mass spectra of the unknown (A) and the TCH346 drug substance (B).

HPLC conditions. The initial conditions consisted of 10% acetonitrile/90% HPLC-grade water (Aldrich, Milwaukee, Wisconsin, USA) containing 0.1% formic acid. A flow rate was set at 1.0 ml/min through a Symmetry C18 4.6-mm i.d. 250-mm length 3.5 μm particle size HPLC column (Waters Corporation, Milford, MA, USA) and under the column temperature of 40 °C. The mobile phase composition was changed linearly to 50:50 within the first minute. From 1 to 10 min, another linear mobile phase gradient to 100% acetonitrile was performed, during which the peak of interest eluted. The post-column water makeup flow was 3.0 ml/min, for a total combined flow of 4.0 ml/min through the trapping cartridge.

NMR conditions. NMR spectra were recorded in at 300 K using a Bruker Avance 600 MHz (125 MHz for ^{13}C) spectrometer equipped with a Bruker CryoPlatform and a 3 mm cryo-fit dual inverse probe fitted with a 60 flow cell (30 μl active volume). The temperature of the probe and amplifier was 15 K. Chemical shifts were referenced to the respective $CDCl_3$ resonances.

The 1D proton spectrum was recorded in 16 scans using a 32 k transient. The 1D ^{13}C spectrum was obtained using 16 k scans of a 64 k transient and a relaxation delay of 4 s. The gs-COSY spectrum was obtained with an F_2 spectral width of 11 ppm in 2 k data points for 128 t_1 increments and 64 scans per t_1. The gs-HMQC spectrum resulted from 256 × 4096 data matrix size

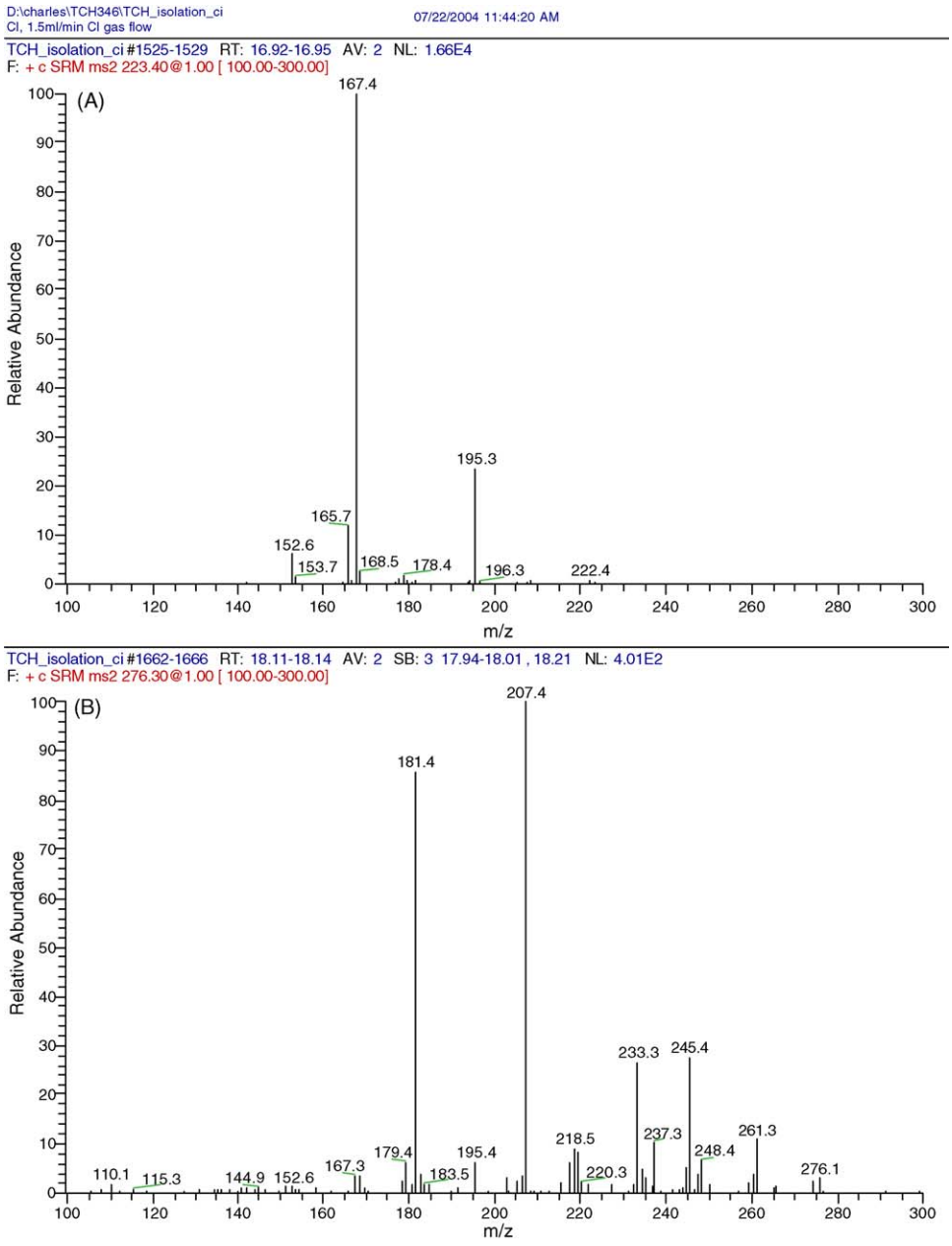


Fig. 5. MS/MS spectra of *m*/*z* 223, the protonated molecular ion of the unknown (A) and *m*/*z* 276, the protonated molecular ion of the TCH346 drug substance (B).

with 64 scans per t_1. The gs-HMBC spectrum resulted from a 128 × 1024 data matrix size with 128 scans per t_1.

3. Results

3.1. LC/MS

Fig. 1 shows the chromatograms of a TCH346 tablet stability sample obtained from four different HPLC methods. Under the acidic mobile phase of 0.1% TFA, the peak of interest (labeled as unknown) was eluted at 11 min in the chromatogram shown in Fig. 1A. No protonated molecular ion was observed for this unknown peak using either an ESI (+) mode or an APCI (+) mode. A negative electrospray ionization ESI mode was then investigated under neutral and basic mobile phase conditions (Fig. 1C and D), No ionization was observed for this peak either. The above results imply that this compound has no ionizable functional groups as it could not be ionized in either a positive or a negative ionization mode. Fig. 1 also demonstrates that the retention times of this unknown peak remain almost constant under all four different mobile phase conditions from pH 2.5 to 8.7. The independence of retention time from mobile phase pH clearly indicates that the charge status of the unknown is not changed from pH 2.5 to pH 8.7. In contrast, the TCH346 drug substance shows a strong dependence of the retention time on the mobile phase pH. The drug substance is a basic compound with a pK_a of 6.3. When the mobile phase was changed from acidic pH to

basic pH, the drug substance was changed from a protonated form to a neutral state, and therefore retained longer in the column.

Fig. 2 shows the UV spectra of the drug substance and the unknown. Compared to the drug substance, the wavelength at maximum absorbance of the unknown was shifted to a longer wavelength (from 283 to 303 nm). This "red shift" indicates that the molecular structure of this compound has a higher conjugation than the drug substance.

The LC/MS studies provide some important information on this unknown even though its molecular weight could not be determined by atmospheric pressure ionization. This unknown is a neutral compound with a higher conjugation than that of the drug substance. In order to determine the structure of this

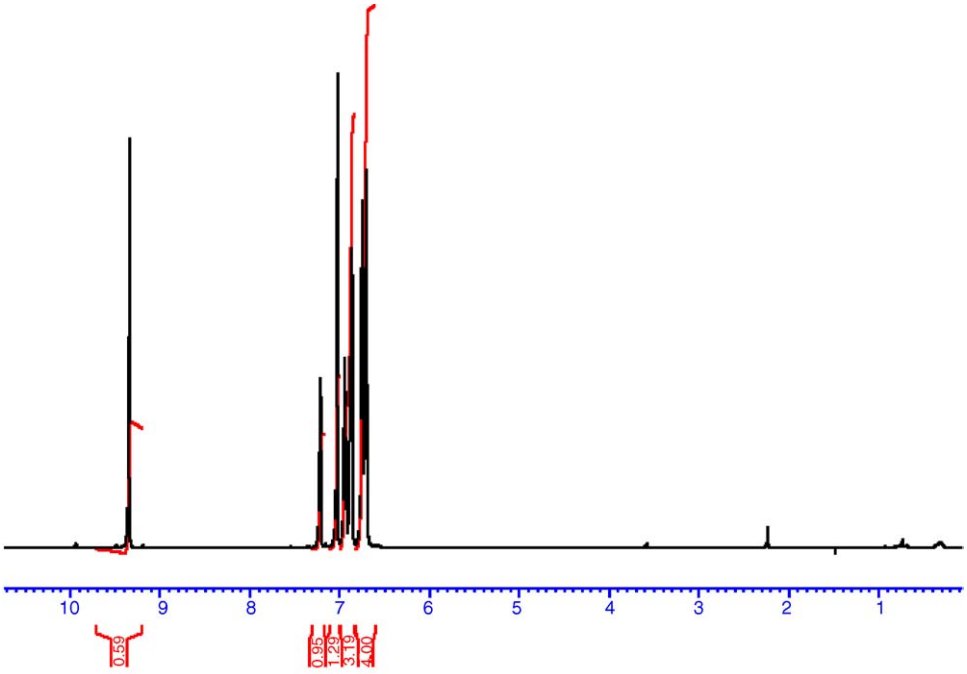


Fig. 6. ^{1}H NMR spectrum of the isolate purified by a second, automated LC/SPE step.

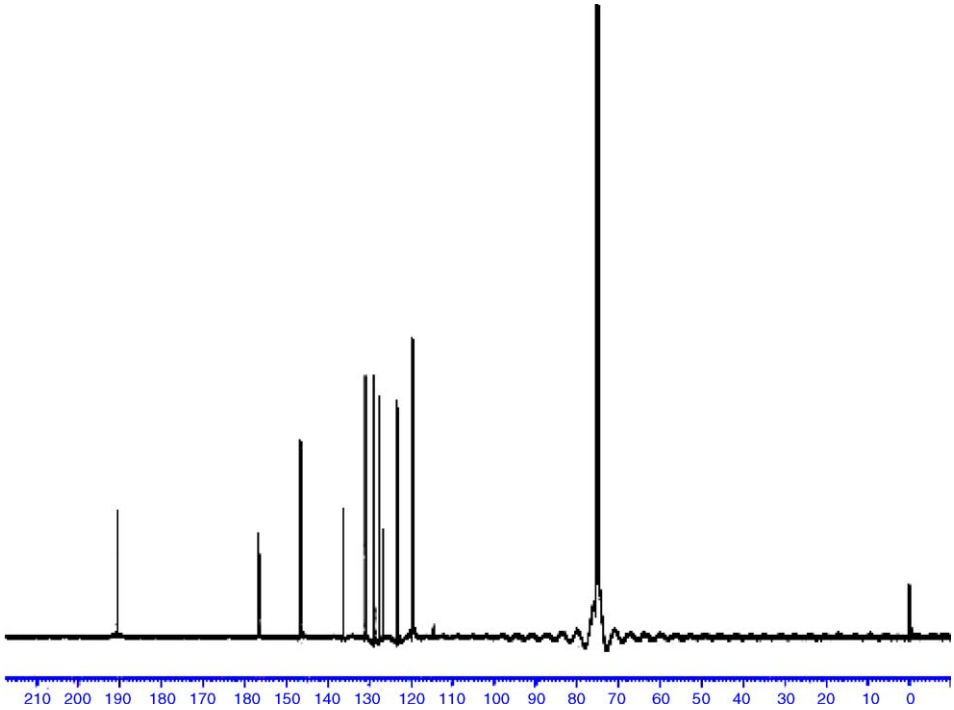


Fig. 7. ^{13}C NMR spectrum of the isolate purified by a second, automated LC/SPE step.

compound, the peak of interest was isolated by multiple LC-fraction collections. The isolate with 80% purity was then tested by other analytical techniques.

3.2. Isolation

Due to the presence of many TCH346 impurities in the TCH346 drug substances, chromatographic separation between the target unknown and the TCH346 impurities became challenged to achieve a good separation required for HPLC fraction collection. According to the LC/MS study, the target unknown is a neutral compound while most of the TCH346 impurities are basic amines, similar to the TCH346 drug substance. In addition, the unknown compound with a high conjugation has a unique UV spectrum compared to other TCH346 impurities. Therefore, the peak of interest could be readily monitored in the HPLC method development. A new HPLC method was developed for isolation, in which perchloric acid was used as a low-pH mobile phase modifier to increase the retention and peak symmetry of protonated basic molecules [21]. The modification of the mobile phase greatly improved the separation between the neutral unknown compound and other basic TCH346 impurities. The effect of anionic additive (in the mobile phase) on the retention time of the basic compound could be due to the combination of the Hofmeister effect and the ionic interaction between anionic additive and basic impurities [21,22].

3.3. GC/MS

Since the unknown could not be ionized by atmospheric pressure ionization techniques (ESI and APCI), the isolate was analyzed by chemical ionization, a much less selective ionization mode. Because the TCH346 drug substance is semi-volatile and can be detected by GC/MS, the unknown may be amenable to this technique as it is a degradation compound of the drug substance. Fig. 3 shows the gas chromatogram of the isolated material. The major peak elutes at 16.9 min and is presumed to be the unknown compound. A minor peak at 18.1 min is due to the TCH346 drug substance resulting from the peak tailing in the chromatogram during the isolation process. The methane chemical ionization spectrum shown in Fig. 4A indicates that the molecular weight of the unknown compound is 222 dalton, confirmed by the presence of $[M+H]^+$ ion of m/z 223 and an adduct ion $[M+C_2H_5]^+$ of m/z 251. The CI-MS/MS daughter ions of the protonated pseudo-molecular ion is shown in Fig. 5.

3.4. LC/SPE/NMR

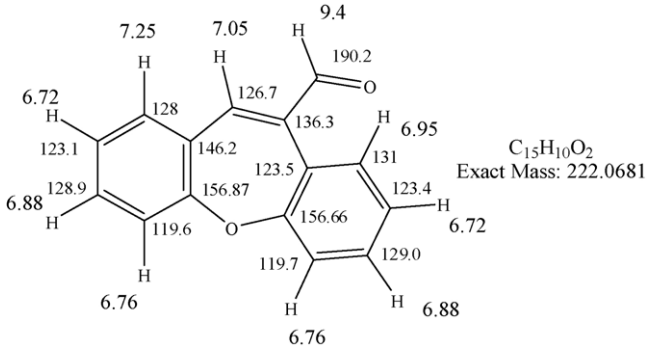
The isolate was dissolved in acetonitrile and injected onto a C18 LC column for purification and to focus the material into the LC/NMR flowprobe. The concentrated analyte in the flowprobe was of sufficient purity and quantity to provide both ^1H and ^{13}C data, as well as 2-D homonuclear and heteronuclear polarization transfer spectra.

Fig. 6 shows the proton ^1H spectrum obtained. There is a single resonance for the aldehyde proton at 9.4 ppm, and 9 aromatic protons. The integration for the aromatics is slightly high due to the presence of the residual chloroform signal under the analyte signal.

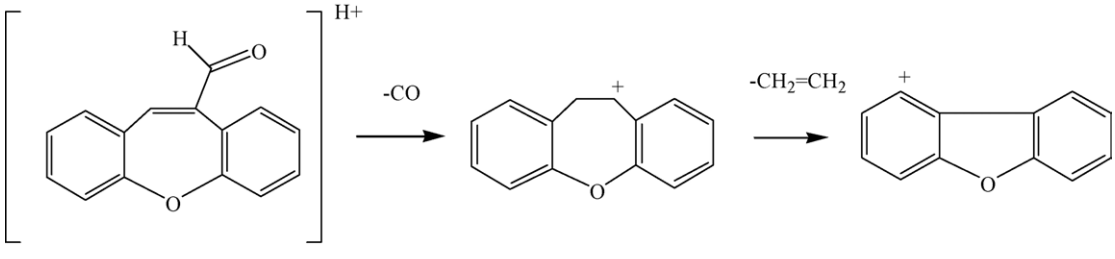
Fig. 7 shows the ^{13}C spectrum. 2-D multinuclear experiments (supplied as supplemental data) were used to make tentative assignments. The six upfield protons (three sets of two) correspond to the six-member ring protons closest to the ether. The resonances within each pair (on opposite rings) are not resolved, so assignments of proton or carbon shifts to particular rings are tentative. The three downfield protons correspond to the three protons closest to the aldehyde.

4. Discussion

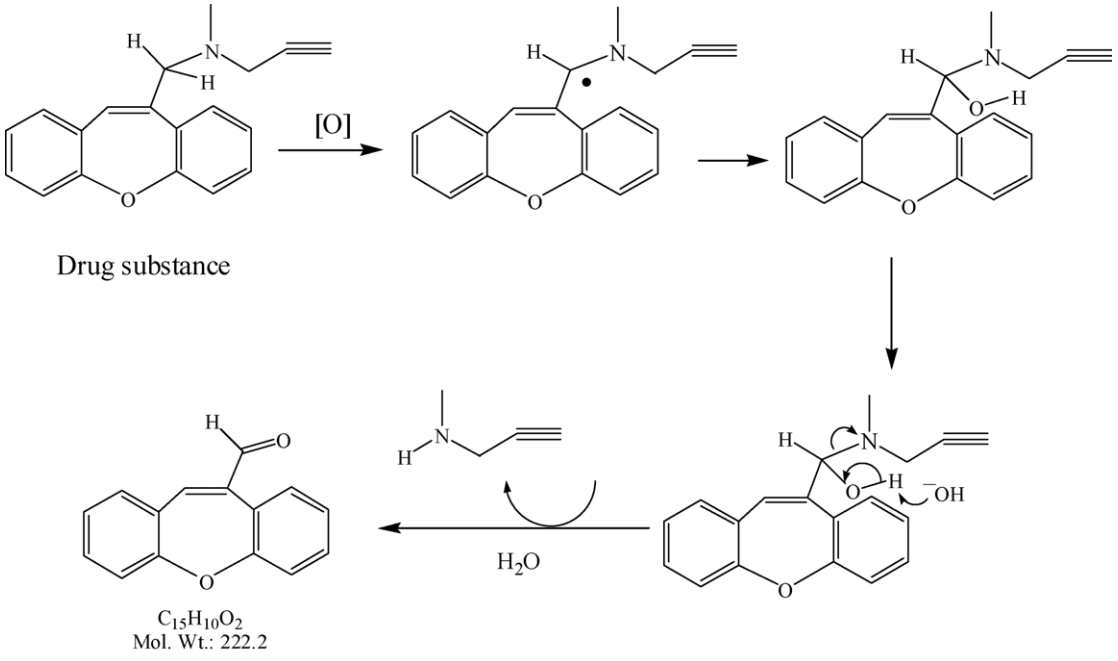
The spectra are consistent with aldehyde structure shown in below.



The compound is a degradation product of TCH346 drug substance. The proposed structure is consistent with the previous observation that it gives no response in electrospray ionization and atmospheric pressure chemical ionization in either positive or negative ionization mode. The formation of aldehyde conjugates the carbonyl double bond to the aromatic ring, resulting in a "red shift" in its UV spectrum compared to the drug substance. The aldehyde structure is also in agreement with the gas phase ion fragmentation obtained by MS/MS.



The degradation pathway for this compound is proposed below.



A reference compound has been synthesized in house. The authentic compound matches the "unknown" in LC retention time and UV spectrum. With this authentic compound, the relative response factor was obtained and the peak can now be quantitatively measured at the wavelength of 281 nm.

5. Conclusions

This study combines various hyphenated techniques to identify an unknown in the TCH346 drug product. LC/UV/MS is used to determine the chemical property of this unknown: a low polarity compound with a highly conjugated structure. Fraction collection was then conducted to isolate relatively pure materials of the unknown for GC/MS and LC/NMR studies. Using GC/MS, the molecular weight and the fragmentation ions were determined under CI MS/MS. The isolated material was further purified using LC/SPE, from which good quality NMR data was obtained. The NMR data characterize the detailed structure of this compound. Using different hyphenated analytical techniques, complimentary information can be obtained. The unknown peak in the TCH346 drug product stability samples has been identified and confirmed as an aldehyde degradation product.

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