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Short communication

Effect of incomplete removal of the *tert*-butoxycarbonyl protecting group during synthesis of a pharmaceutical drug substance on the residual solvent analysis

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ABSTRACT

During development of the residual solvent method using headspace-GC for a drug substance, an unexpected peak was observed in the chromatography. GC–MS analysis confirmed the unknown peak identity as isobutylene. An understanding of the source of the isobutylene was required in order to develop appropriate impurity and residual solvent control strategies for the drug substance. The experiments performed to determine the source of the isobutylene peak observed in the headspace-GC chromatography and how the *tert*-butoxycarbonyl (BOC) de-protection step used in the drug substance synthesis contributes to its observation are discussed.

1. Introduction

Manufacturing of pharmaceutical drug substances requires strict control of impurities and solvents. Impurities may be present in the raw materials used for synthesis, formed as by-products during the manufacturing process, or arise as degradation products during storage over time. Solvents used in the process are often not totally removed by practical manufacturing techniques, and consequently, low levels can be present in pharmaceuticals. Acceptable levels of impurities and residual solvents are included in regulatory guidance documents, in particular in guidelines Q3A and Q3C issued by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). The analysis of impurities and residual solvents is an essential part in the control strategy for drug substances used in preclinical or clinical trials, as well as for use in commercial drug products.

A *tert*-butoxycarbonyl (BOC) fragment is frequently used in the synthesis of organic molecules to protect amine groups, and is typically removed under acidic conditions [1]. The generally accepted mechanism for BOC de-protection [2] is shown in Fig. 1. Protonation of the carbonyl oxygen results in degradation to initially produce the unprotected amine, carbon dioxide, and a highly reactive *tert*-butyl cation. The *tert*-butyl cation can then decompose to isobutylene unless it is trapped by a suitable reagent.

An understanding of the by-products from a BOC de-protection can be necessary in order to develop appropriate control strategies for a drug substance. There are limited published studies on the by-products of BOC de-protection and its implications in pharmaceutical development. This paper describes the experiments performed to understand how the BOC de-protection step contributes to the unexpected observation of an isobutylene peak in the headspace-GC chromatography method used for residual solvent analysis of a drug substance material.

2. Experimental

2.1. Chemicals

The water used was de-ionized with a Milli-Q Gradient A10 system from Millipore (Cambridge, ON). The t-butyl ethyl ether, t-butanol, t-butyl chloride, N-boc pyrrolidine, trifluoroacetic acid (TFA), and DMSO were supplied by Sigma–Aldrich (Oakville, ON). Methanol was supplied by EMD (NJ, USA). The drug substance and its precursors and filtrates were from Eli Lilly and Company (Indianapolis, IN, USA).

2.2. Headspace gas chromatography

An Agilent Technologies 6890N GC system equipped with Agilent G1290B Headspace Sampler and Flame Ionization Detector

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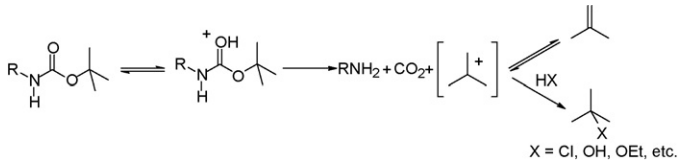

Fig. 1. Mechanism for BOC de-protection.

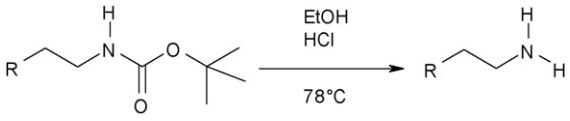

Fig. 2. BOC de-protection step used in the synthesis of drug substance.

(FID) was used. The headspace-GC method developed for the residual solvent analysis of the drug substance uses an Agilent DB-624 capillary column (6% polycyanopropylphenylsiloxane–94% polydimethylsiloxane, 30 m × 0.32 mm i.d., 1.8 μm film thickness), and the instrument conditions are outlined in Table 1.

2.3. High performance liquid chromatography with mass spectrometric detection

An Agilent 1100 HPLC system equipped with a UV detector was used. The HPLC method developed for the analysis of the drug substance and its impurities uses a reversed phase column (Zorbax Bonus RP, 4.6 mm × 75 mm, 3.5 μm particle size, Agilent) with gradient elution. Mobile phase A consists of 5/95/0.1 (v/v/v) methanol, water, and TFA and mobile phase B consists of 95/5/0.1 (v/v/v) methanol, water, and TFA. The gradient increased linearly from 0% to 100% B in 26.7 min and was held at 100% B until 32 min. A linear gradient to 0% B occurred until 32.1 min, and this was held until 37 min. UV detection was carried out at 228 nm and the flow rate was 1.5 mL/min. Mass spectral analyses were carried out on an LCQ Deca ion trap mass spectrometer (Thermo Electron, ME, USA) with an APCI ionization source in the positive mode and scan range 50–600 *m*/*z*. The mass spectrometer was set with a source heater temperature of 450 °C; a capillary temperature of 250 °C and voltage of 3.00 V; sheath and auxiliary gas flow rates of 40 and 10 arbitrary units, respectively; a tube lens offset of −25.00 V; and an APCI source current of 5.00 μA.

Table 1
GC instrument conditions.

FID system	
6890N GC	
Injection port	Volatiles interface
Temperature	140 °C
Split ratio	1:10
Carrier gas	Helium
Inlet pressure	10.0 psi
Detector	FID, 250 °C
Column flow	2.1 mL/min (typical value)
GC oven program	
Initial temperature	45 °C
Initial time	5.0 min
Rate	10 °C/min
Final temperature	175 °C
Final time	1.0 min
Column	30 m × 0.32 mm × 1.8 μm DB-624
G1290B Headspace Sampler	
Vial pressure	2.6 psi
Headspace oven	85 °C
Loop temp.	95 °C
Transfer line temp.	130 °C
Equilibration time	0.1 min
GC cycle time	35.0 min
Pressurization	0.5 min
Vent (loop fill)	0.2 min
Inject	1.0 min

2.4. Accurate mass LC–MS

An Agilent 1100 HPLC system equipped with a PDA detector was used. The HPLC method developed for the analysis of the drug substance and its impurities uses a reversed phase column (Zorbax Bonus RP, 4.6 mm × 150 mm, 3.5 μm particle size, Agilent) with gradient elution. Mobile phase A consists of 0.1% TFA in water and mobile phase B consists of 0.1% TFA in methanol. The gradient varied linearly from 5% to 95% B in 25 min and was held at 95% B until 30 min. A linear gradient to 5% B occurred until 30.1 min, and this was held until 35 min. UV detection was carried out in the range of 200–400 nm and the flow rate was 1.2 mL/min. Mass spectral analyses were carried out on Finnigan LTQ FT 6T mass spectrometer (Thermo Electron Corp.) in positive ion electrospray ionization mode and scan range from 100 to 1000 *m*/*z*. The mass spectrometer was set with a capillary temperature of 200 °C and source voltage of 4.05 V; capillary voltage of 39.0 V; sheath gas flow rate of 40 arbitrary units; a tube lens voltage of 98.00 V.

2.5. Synthesis of isobutylene precursor

100 mg of the intermediate containing a BOC functional group was combined with 1.0 mL of ethanol and 0.3 mL of 5 M HCl solution and mixed at 40 °C for 4 h. After cooling to room temperature, the mixture was vacuum filtered and washed with alcohol reagent. The precipitate was transferred to a watch glass and left to air-dry overnight.

3. Results and discussion

During the development of the residual solvent method for a new drug substance, an unknown and unexpected peak was observed in the chromatography. GC–MS analysis confirmed the unknown peak identity as isobutylene. During the synthesis of this drug substance, a BOC fragment is used as a protecting group and the BOC de-protection step was confirmed to be the source of the impurity. The BOC protecting group is removed using acidic conditions and reaction completion is monitored by HPLC analysis before forward processing the material to the final step. Since it is not possible that isobutylene could carry over into the final drug substance material (it is a gas at room temperature), an investigation into the source of the isobutylene peak observed was initiated.

3.1. Investigation into conversion of de-protection by-products to isobutylene

The BOC de-protection step used in the synthesis of the drug substance is shown in Fig. 2. When the *tert*-butyl cation is formed during BOC de-protection, it can decompose to isobutylene or be trapped by a suitable reagent. Based on the components of the reaction mixture, t-butanol, t-butyl ethyl ether, and t-butyl chloride are all possible by-products of the de-protection and these could convert to isobutylene under the GC headspace conditions. Drug substance samples were spiked with t-butanol, t-butyl ethyl ether, and t-butyl chloride to observe any change in the isobutylene peak area when analysed using the GC headspace condition. If the isobutylene peak area remains the same after spiking the solvent, this solvent does not convert to isobutylene during GC analysis.

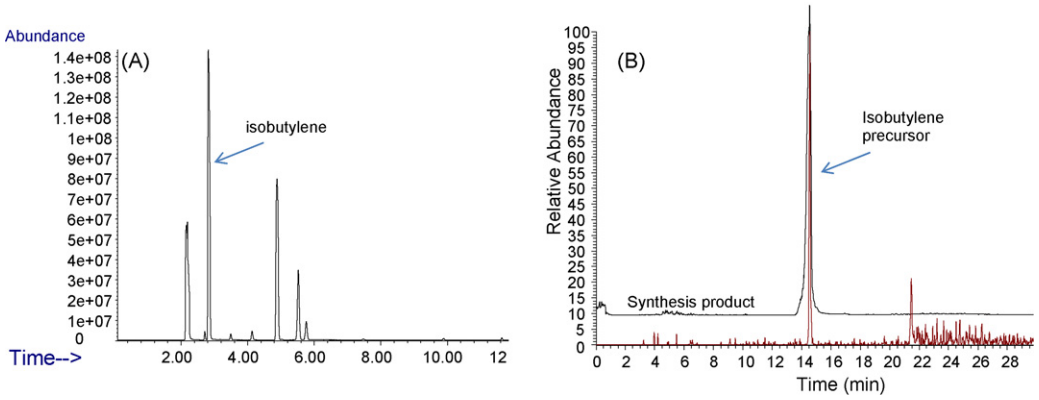

Fig. 3. GC-headspace (A) and LC–MS (B) analyses of isobutylene precursor.

As the levels of t-butanol were increased from 0.015% to 1.5%, the isobutylene peak area remained the same. Further, the t-butanol peak areas were equivalent for corresponding spike levels into DMSO compared to spikes into the samples containing drug substance and DMSO. The same result was observed for the samples containing spikes of t-butyl ethyl ether. As the levels of t-butyl chloride spikes were increased from 0.015% to 1.5%, the isobutylene peak area increased. However, the t-butyl chloride peak areas were equivalent for corresponding spike levels into DMSO compared to spikes into the samples containing drug substance and DMSO suggesting that the t-butyl chloride does not convert to isobutylene under the headspace-GC conditions. In addition, an isobutylene peak was observed when t-butyl chloride-spiked DMSO was analyzed. Based on this, it can be inferred that an impurity that converts to isobutylene was present in the t-butyl chloride used for spiking and would explain the increase in isobutylene peak area with increased spiking level. This experiment provides evidence that neither t-butanol, nor t-butyl ethyl ether, nor t-butyl chloride is the source of the isobutylene peak seen in the GC analysis of the drug substance.

3.2. Determination of whether the source of isobutylene is BOC-protected

N-boc pyrrolidine was used to assess whether BOC de-protection and observation of an isobutylene peak can occur under the headspace-GC conditions to provide information on the structure of the precursor. Indeed, a sample of N-pyrrolidine analyzed by headspace-GC showed an isobutylene peak in the chromatography indicating that the precursor could be BOC protected.

Concurrent to this investigation, the BOC de-protection step for the synthesis of the drug substance was modified in an attempt to remove the isobutylene precursor. The key modification was the addition of an ethanol and water re-crystallization prior to forward processing. The drug substance synthesized using the modified route did not show an isobutylene peak in the headspace-GC chromatogram suggesting that the precursor was removed using the modified synthesis.

3.3. Comparison of impurity profiles

The two separate drug substance lots (synthesized using the original and modified BOC de-protection step) were analyzed using HPLC-UV and their impurity profiles compared. With injection of a 0.1 mg/mL nominal sample concentration, the impurity profiles were identical. Subsequent to this experiment, samples at ten times the nominal concentration were prepared to see if there were any differences in low level impurities (<0.02%) not detected at the nominal concentration. A small impurity peak at 14.4 min was observed in the original drug substance lot that was not observed in the drug substance lot prepared with the additional re-crystallization step. The structure was confirmed by accurate mass LC–MS to be a product of the de-protection with the BOC group still attached.

3.4. Confirmation of isobutylene precursor

LC–MS analysis of filtrates from the BOC de-protection step (original and modified) showed the presence of the impurity. Analysis of the product from the original BOC de-protection step showed the impurity peak (by LC–MS) and the isobutylene peak (by headspace-GC), whereas the impurity peak and isobutylene peak were not observed in the analysis of the product from the modified BOC protection step. This suggests that the impurity is the precursor to isobutylene in the headspace-GC analysis of the drug substance.

The impurity was synthesized (see Section 2.5) and analyzed using headspace-GC and LC–MS in order to verify the structure of the impurity. The chromatography from each analysis is shown in Fig. 3. The synthesized precursor eluted at the retention time originally observed for the impurity peak and produced a large isobutylene peak in the headspace-GC analysis. Note that the other peaks present in the GC-headspace analysis (Fig. 3A) are residual solvents from the synthesis of the precursor. Therefore, a product from the incomplete removal of the BOC protecting group during the de-protection step was the precursor to isobutylene in the residual solvent analysis.

3.5. Implications to control strategy

It is worthy to note that it was a trace amount of the impurity that produced a sizeable isobutylene peak in the residual solvent analysis. This impurity was not detectable using the nominal sample preparation for the existing HPLC-UV impurities method. Based on the low level of the impurity, it did not need to be incorporated into the drug substance control strategy. Further, the synthetic route was modified to include a new re-crystallization step, so the precursor is now removed in the synthesis prior to forward processing.

4. Conclusion

A series of experiments were used to identify the source that lead to the unexpected production of isobutylene during the

headspace-GC analysis of a drug substance. The source was determined to be an impurity that resulted from the BOC de-protection step that had the BOC protecting group still attached. Although only a trace amount of this impurity was present in the final drug substance (<0.01%), it caused a sizeable (>0.1%) isobutylene peak in the GC analysis. Isobutylene was an artifact peak of the headspace-GC analysis and sizeable levels were observed because of its high vapour pressure in headspace analysis.

In general, samples containing BOC functional groups are susceptible to losing the BOC group and forming isobutylene under headspace-GC conditions. It is therefore important to ensure complete removal of the BOC protecting group during drug substance synthesis, especially if the de-protection step occurs near the end of the synthetic route. This investigation shows the importance of understanding the limitations of existing methods and modifying them to aid in the identification of unknown peaks. Additionally, awareness of the by-products of a BOC de-protection during synthesis may be necessary in order to understand the characterization results of the final product.

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