Journal of Pharmaceutical and Biomedical Analysis 92 (2014) 127–134

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Determination of 2-Hydroxypyridine-1-Oxide (HOPO) at sub-ppm levels using derivitization and gas chromatography with mass spectrometry detection (GCMS)

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ARTICLE INFO

Article history:
Received 18 October 2013
Received in revised form 2 January 2014
Accepted 11 January 2014
Available online 21 January 2014

Keywords:
2-Hydroxypyridine-1-Oxide (HOPO)
Derivitization
Gas chromatography
Mass spectrometry quantitification
Validation
Trace analysis

ABSTRACT

This work describes the development and validation of an analytical method to determine residual trace levels of 2-Hydroxypyridine-1-Oxide (HOPO) in an active pharmaceutical ingredient (API). A method was required to be specific and sensitive enough to determine sub-ppm levels of this reagent. The approach taken to use a derivitization step overcame two of the primary challenges associated with the analysis of HOPO. Firstly, HOPO can tautomerize and the derivitization step provides a single stable entity to monitor, and secondly, the reaction enhances the volatility of the analyte to facilitate the use of gas chromatography. Mass spectrometry detection provides both suitable specificity and sensitivity. This paper describes the method development and optimisation of the derivitization step, the chromatographic conditions and mass spectrometry detection, together with a summary of the validation of the method. The method has been demonstrated to be robust and suitable to determine HOPO levels in commercially manufactured API materials.

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1. Introduction

2-Hydroxypyridine-1-Oxide (HOPO) is used as a coupling reagent to catalyze the formation of amide bonds within a specific commercial API process at Bristol-Myers Squibb Pharmaceuticals (BMS). HOPO promotes this reaction by forming an active ester intermediate which then reacts via S_NN mechanism to form the amide bond. The HOPO catalyst is regenerated upon formation of the API.

A genotoxic impurity risk assessment had originally indicated there was no need to control HOPO levels within API as it had presented negative in *in-silico* assessment and was reported to be non-mutagenic [1]. However, an Ames test was conducted on HOPO at BMS following concern that similar compounds were mutagenic. An Ames study [2–4] was conducted with *Salmonella typhimurium* strains TA100, TA98, TA1535 and TA537 and *Escherichia coli* WP2 uvrA using both the plate incorporation and pre-incubation methods in accordance with Good Laboratory Practices (GLP) [5,6] and applicable regulatory guidelines [7,8]. Mutagenic activity was observed with strain TA100 in the plate incorporation and pre-incubation assays in the presence of an induced rat liver microsomal fraction (S9) and with *E. coli* WP2 uvrA in the pre-incubation assay in the absence of S9 metabolic activation.

Although these Ames results were not sufficient to reclassify HOPO as a genotoxic impurity, BMS chose to categorize it as a potential genotoxic impurity due to its use in commercial product manufacture. Subsequently, HOPO was required to be controlled at the threshold of toxicological concern (TTC). Furthermore, the US Food and Drug Administration (FDA) recommends an additional safety factor for pediatric populations [9], which lowered the HOPO control level down to the sub-ppm level. A limit of 0.2 ppm for residual HOPO in API was imposed on all material prepared from this specific synthesis.

An analytical method was required which overcame the sensitivity challenges of monitoring a potential genotoxic impurity at sub-ppm levels, as well as the analytical challenges associated with the direct detection of HOPO due to its polar nature and tendency to tautomerize between keto and enol forms in solution, Fig. 1.

2. Materials and equipment

2.1. Chemicals

HOPO (2-Hydroxypyridine-1-oxide) >98% was obtained from Aldrich, Dorset, Great Britain. The dissolution solvent, Pyridine 99.9% was obtained from VWR AnalaR, Lutterworth, Great Britain. Derivatisation reagent, MtBSTFA + 1% tBDMCS

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http://dx.doi.org/10.1016/j.jpba.2014.01.009

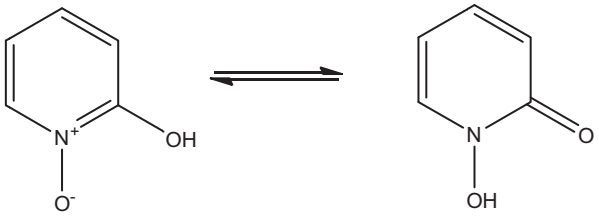


Fig. 1. Tautomerization of HOPO.

(N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-Butyldimethylchlorosilane) >95% was obtained from Aldrich, Dorset, Great Britain.

2.2. Gas chromatography gases

Helium, 99.999% was obtained from Air Liquide™ for use as the carrier gas. Hydrogen, 99.9999% and Zero-Air, <0.05% total hydrocarbon content, for the fuel and oxidiser flows respectively were generated using a Parker FID-1000UK station.

2.3. Equipment

An Agilent 6890 capillary gas chromatograph (GC) equipped with a split inlet coupled to an Agilent 5973N Mass Selective Detector (MSD) was used for analysis. The injection port liner was an Agilent 4 mm internal diameter, open glass tube packed with fused silica wool. The derivitization reaction was carried out in Agilent 2.0 mL borosilicate glass injection vials, 12 mm × 32 mm with Teflon™ lined rubber septa in aluminum crimp caps. Solutions were transferred into these vials using Thermo Fisher Scientific electronic Finnpipettes, 100–1000 μL. For the derivitization reaction an IKA Works Inc MS2 Minishaker vortex agitator, 200–2500 rpm and Stuart block heater, SBH200DC were employed.

3. Results and discussion

3.1. Method development

HOPO is a difficult species to analyse directly due to its physical properties. It is highly polar, which presents problems for GC analysis due to limited volatility, and HPLC analysis by having little or no retention on the majority of HPLC reversed-phase columns. In instances when HOPO is retained, the peak shape is compromised by the on-column equilibrium between the keto and enol forms. Peaks attributable to HOPO tend to be shouldered and exhibit unacceptable tailing. Hence, this method required a mechanism by which HOPO would be retained in one form.

Initially, reversed-phase LC with UV detection was investigated using stationary phases specific for polar species as well as hydrophilic interaction chromatography (HILIC). Although attempts to overcome the on-column tautomerization using phosphate buffer and careful manipulation of the mobile phase pH were unsuccessful, it was found that the addition of ion pairing reagent, trifluroroacetic acid (TFA) to eluents comprising of acetonitrile and water, significantly improved peak shape and reproducibility. Combining this with a comprehensive pre-injection conditioning and equilibration procedure, peak tailing criteria also fell within acceptable limits. The chromatographic separation was achieved using a Waters Atlantis T3 column, 150 mm length × 4.6 mm internal diameter, 3 μm particle size. The detection limit (DL) for UV detection of HOPO was estimated at 20 ppm with respect to the API and subsequently was not sufficiently sensitive for trace level analysis. In addition, the LC conditions developed were not suitable for coupling with mass spectrometry detection. Therefore, other approaches were evaluated.

Use of GC was explored due to the potential to achieve a cleaner chromatographic separation where only the more volatile components of the sample matrix would be introduced on-column, thus providing the option to significantly increase the sample concentration and subsequently enhance the relative response of the analyte. The original approach was to develop a rapid GC separation using an Agilent HP-5MS column (30 m length × 0.25 mm internal diameter × 0.25 μm film thickness) that would not distinguish between the keto and enol forms of HOPO, allowing them to be quantified as one peak. As HOPO has limited volatility, the GC inlet temperature was set to 280 °C and a solution equivalent to 300 ppm of HOPO with respect to the API in dimethylsulfoxide (DMSO) was injected. No peak(s) attributable to HOPO was observed using flame ionisation detection (FID) so the inlet temperature was increased to 325 °C and the sample re-injected. This time, although a peak response for HOPO was observed, the chromatography was poor and the peak shape indicated the presence of both tautomers. As a result, the method development direction turned to the use of derivatization to enhance the analytical properties of HOPO as well as to provide a single, stable entity for detection.

N-tert-Butyldimethylsilyl-N-methyltrifluroroacetamide with 1% tert-Butyldimethylchlorosilane (MtBSTFA + 1% tBDMCS) is a derivatization reagent often used for GC analysis. MtBSTFA + 1% tBDMCS reacts with HOPO to give the butyldimethylsilyl (tBDMS) derivative shown in Fig. 2. Not only does this reaction prevent the tautomerization of HOPO, the derivative species is less polar than HOPO and subsequently more volatile. A variety of different dissolution solvents in which HOPO, the API and the derivatizing agent were sufficiently soluble were assessed but the reaction was only found to proceed in pyridine.

A chromatographic separation of the HOPO-tBDMS derivative from the volatile components of the API was developed using a 100% dimethyl polysiloxane Rtx-1 Crossbonded Restek column (15 m length × 0.32 mm id × 1.0 μm film thickness) and flame ionization detection (FID) at 250 °C. Use of a headspace autosampler was not a viable option due to the boiling point of the pyridine dissolution solvent being lower than that of the HOPO derivative. Therefore direct liquid injection was used to introduce the solutions into the GC inlet. 1.0 μL of sample solution was injected

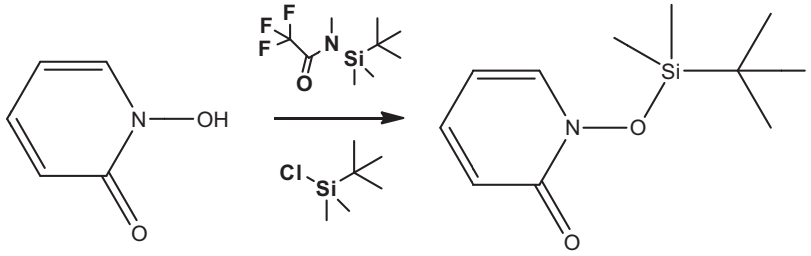


Fig. 2. Derivatization of HOPO.

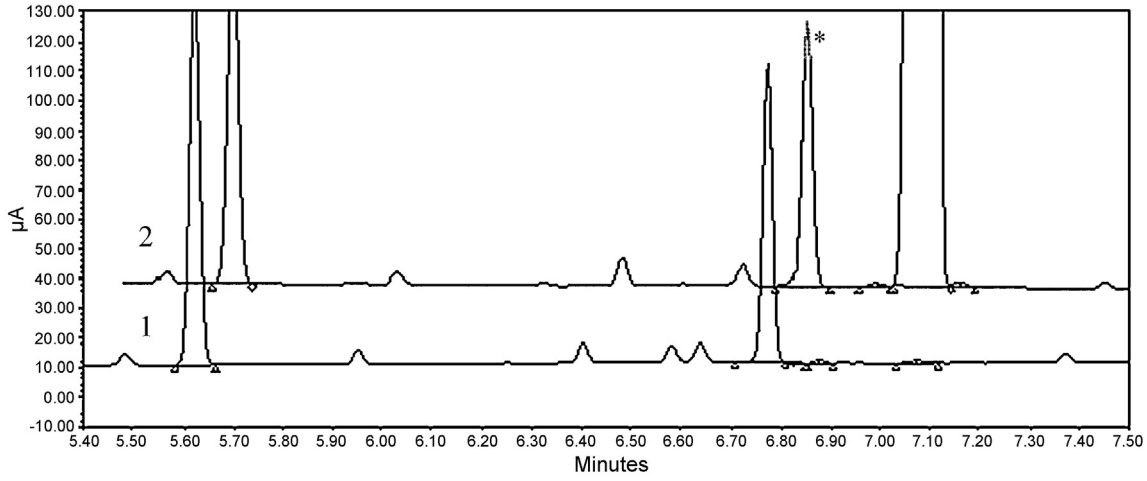
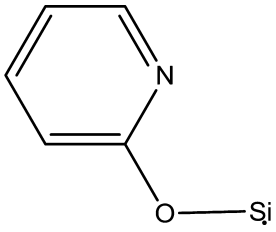
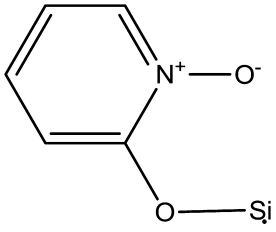
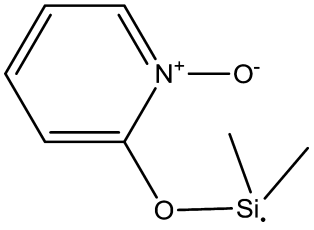
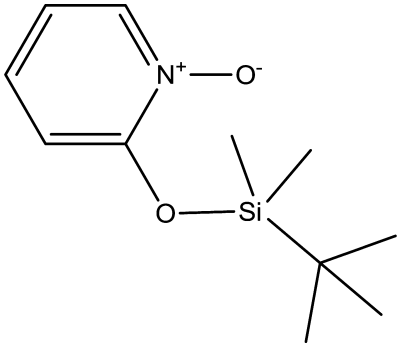


Fig. 3. GC-FID overlay of a HOPO standard and spiked API sample.

into the split port of the GC apparatus maintained at 175 °C using a constant pressure of 10 psig. The split flow was set to 50.0 mL/min. Helium was employed as the carrier gas. The gradient oven program started at an initial temperature of 80 °C which was held for 1 minute then increased at 20 °C/min to 250 °C and held for 3 min. The chromatographic separation for a 0.1 mg/mL HOPO standard and spiked API sample is shown in Fig. 3. The Limit of Detection (LOD) for HOPO using this method was found to be 0.006% (w/w) or 60 ppm with respect to the API. The absolute mass (with respect to dissolution solvent) of derivatized HOPO that can be detected using these method conditions is 1.2 ppm.

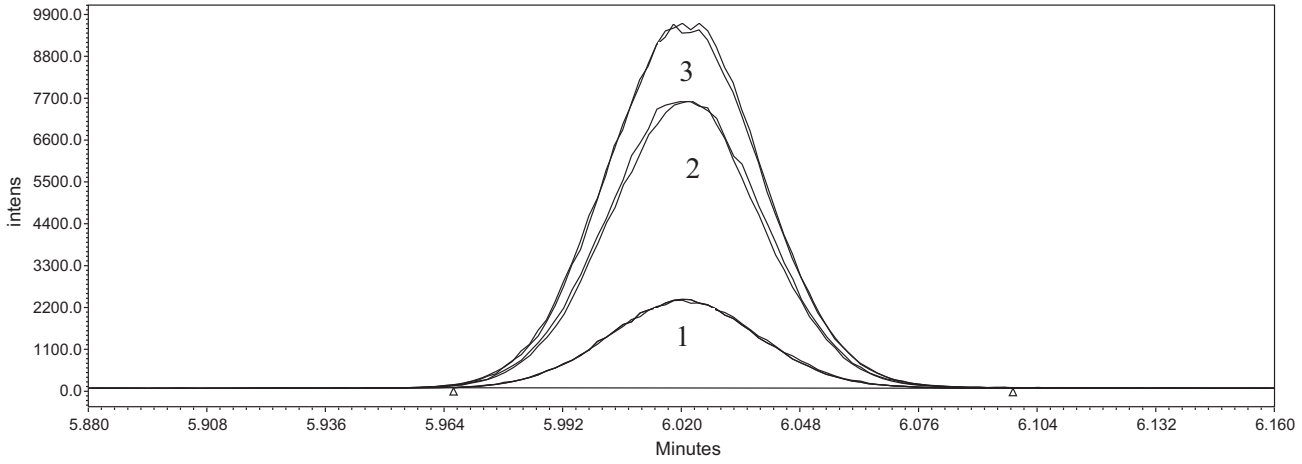
In order to achieve sensitivity at the desired level for HOPO, these GC separation parameters were coupled to a Mass Spectrometry Detector (MSD) using an Electron Impact (EI) source for ionization of analytes. The source temperature was set at 230 °C, emission at 34.6 μA and electron energy at 69.9 eV. A quadrupole temperature of 150 °C was used and the transfer line temperature was set at 310 °C. An initial MS scan between 50 and 300 amu was performed to investigate how the HOPO-tBDMS derivative ionized in the source. HOPO-tBDMS derivative ions were observed at *m*/*z* 122.01, 138.00, 168.05 and 225.12, Table 1. The predominant ion is the *m*/*z* 168.05 fragment. Subsequently, the acquisition mode was changed to SIM (Selective Ion Monitoring) at *m*/*z* 168 to enhance the sensitivity and selectivity of detection.

Table 1
Structures of HOPO-tBDMS derivative ions.

Ion (*m*/*z*)	Structure
122.01	
138.00	
168.05	
225.12	

3.2. Optimization of the derivatization procedure

The derivatization step was developed in 2.0 mL vials that could be introduced directly onto a GC autosampler. This effectively streamlined the procedure and reduced the time prior to sample injection, a benefit given the reactive nature of potential genotoxic impurities. A 3:1 composition ratio of the MtBSTFA + 1% tBDMCS derivitizing agent to dissolution solvent was found to be the optimum quantity required for the reaction. Fig. 4 shows the derivatized HOPO responses of API spiked with 0.2, 1.2 and 2.4 ppm HOPO after incubation at 60 °C for 60 and 75 min. The SIM chromatograms for each concentration are identical. Fig. 5 shows the derivatized HOPO responses of API spiked with 0.2 ppm HOPO after incubation at 30 °C, 45 °C, 60 °C and 75 °C for 60 min. This data indicates that the derivatization reaction has gone to completion after 60 min at 60 °C. The stability data presented in Table 2 demonstrates that no significant chemical degradation of the derivatized



Key:
1 - 0.2 ppm (60 and 75 minutes superimposed)
2 - 1.2 ppm (60 and 75 minutes superimposed)
3 - 2.4 ppm (60 and 75 minutes superimposed)

Fig. 4. SIM overlay of API sample solutions spiked with 0.2, 1.2 and 2.4 ppm HOPO prepared using different derivatization times.

Table 2
24 hour stability data for 0.2 ppm standard and sample solutions.

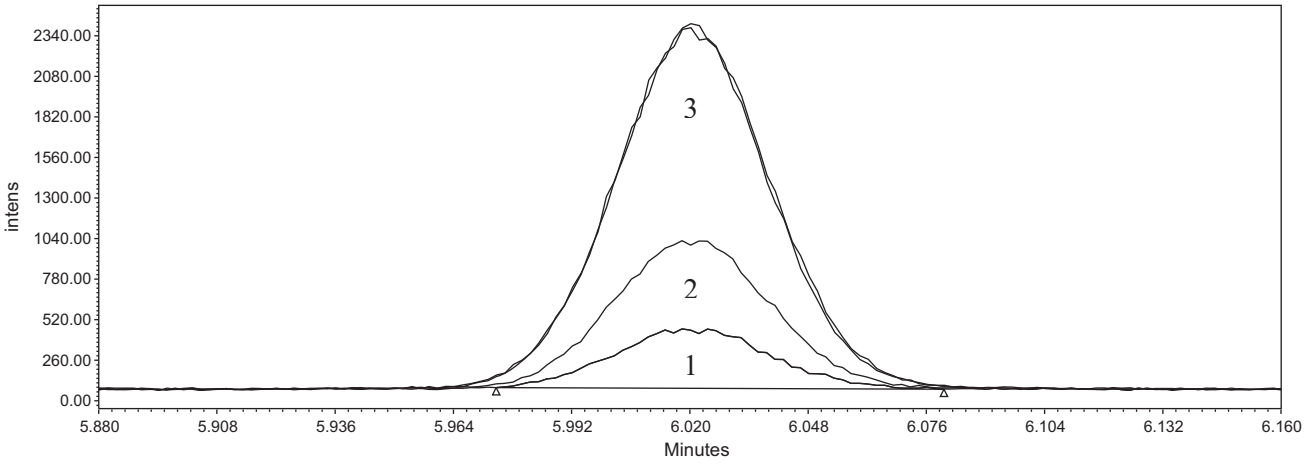
Solution	Preparation	Storage condition	ppm	% Recovery
Standard	1	RT/RL	0.19	95.3
	2	RT/RL	0.19	97.1
Sample	1 (unpierced)	RT/RL	0.50	107.4
	2 (pierced)	RT/RL	0.53	110.4

HOPO occurs within 24 h of sample preparation when stored at Room Temperature/Room Light (RT/RL).

3.3. Final derivatization procedure

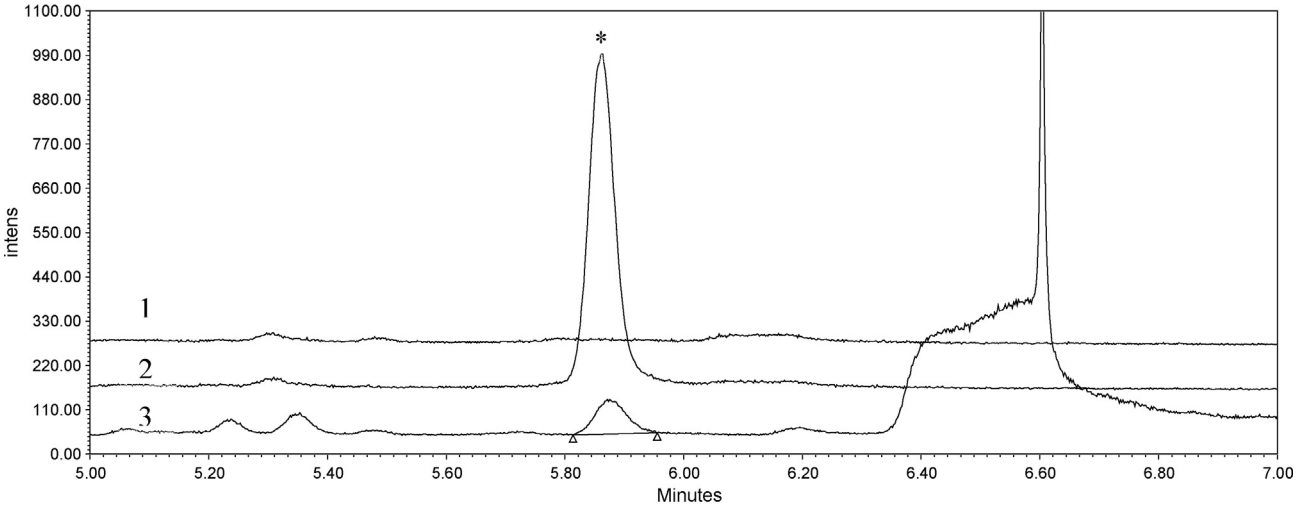
An intermediate standard solution of HOPO was prepared at a concentration of 24 μg/mL in pyridine. 250 μL of this intermediate standard solution and 750 μL of MtBSTFA + 1% tBDMCS derivitizing agent were transferred to a 2.0 mL GC vial to give a sensitivity standard solution at 6 μg/mL (equivalent to 0.2 ppm of HOPO with respect to sample concentration). This was vortexed to mix thoroughly then transferred to a heater block at 60 °C for 60 min. A second intermediate standard solution of HOPO was prepared at a concentration of 140 μg/mL in pyridine. 250 μL of this intermediate standard solution and 750 μL of MtBSTFA + 1% tBDMCS derivitizing agent were transferred to a 2.0 mL GC vial to give a working standard solution at 36 μg/mL (equivalent to 1.2 ppm of HOPO with respect to sample concentration). This was vortexed to mix thoroughly then transferred to a heater block at 60 °C for 60 min. Working standards were prepared in duplicate.

API sample solutions were prepared by weighing 300 mg of API into a 2.0 mL GC vial. 250 μL of pyridine and 750 μL of MtBSTFA + 1% tBDMCS derivitizing agent were transferred to the vial. This was



Key:
1 - 30°C
2 - 45°C
3 - 60°C and 75°C (superimposed)

Fig. 5. SIM overlay of API sample solutions spiked with 0.2 ppm HOPO prepared using different derivatization temperatures.



Key:
1 - Blank Solution
2 - 0.2 ppm HOPO Standard Solution
3 - Sample solution containing HOPO
* - Derivatized HOPO peak at retention time 5.9 minutes.

Fig. 6. SIM overlay of a blank, 0.2 ppm standard and a sample solution.

vortexed until all API had dissolved then transferred to a heater block at 60 °C for 60 min. Blank solutions were prepared by transferring 250 μL of pyridine and 750 μL of MtBSTFA + 1% tBDMCS derivitizing agent to a 2.0 mL GC vial. This was vortexed to mix thoroughly then transferred to a heater block at 60 °C for 60 min. All derivatized solutions were allowed to equilibrate to room temperature before injection. All solutions were stored at RT/RL prior to analysis and injected within 24 h.

3.4. Optimization of the method conditions

When API prepared using an alternative (HOPO free) process was injected, the sample chromatogram contained a peak at the same retention time as HOPO. This peak was then found to be present in succeeding chromatograms including blank injections. A number of experiments were conducted in an attempt to determine the origin of this interfering peak and separate it from HOPO. These included the addition of several blank injections post sample analysis to 'clean' the column, introducing a pre-run column condition, changing the column, changing the needle wash solution to avoid carryover, modifying the oven gradient, modifying the inlet temperature and changing the inlet liner.

It was observed that as the inlet temperature increased so did the response for the interfering peak. This indicated that the interference is being caused by a component of the API that is not volatilised at the time of sample injection but is slowly being removed from the liner with each subsequent injection. The inlet temperature was reduced to 150 °C and the oven gradient was modified. The modified program started at an initial temperature of 80 °C which increased at 20 °C/min to 150 °C and held for 2 min, then increased at 10 °C/min to 250 °C, then increased at 40 °C/min to 300 °C and held for 2 min. Optimization of these parameters successfully separated HOPO from the interfering peak and reduced the response of the interference.

A number of other challenges were encountered during optimisation of the method conditions. The high temperatures and vacuum lead to poor column stability which was overcome by introducing a column pre-conditioning procedure at 320 °C for 1 h before use. Introduction of the high sample concentration into the GC inlet was found to significantly reduce injection port liner lifetime so the glass liner needed to be changed before each analysis. The GC injector required washing with six syringe volumes of pyridine before and after each injection and the pyridine wash vial needed to be changed prior to each analysis to prevent carryover.

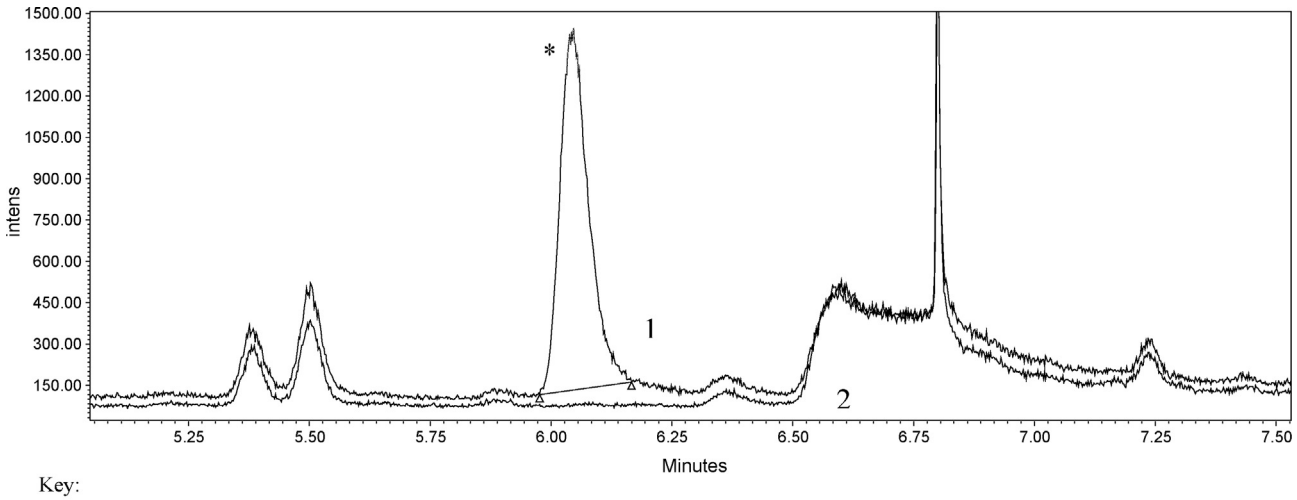
3.5. Final instrument parameters

The final chromatographic separation was achieved using a 100% dimethyl polysiloxane; Rtx-1 Crossbonded Restek column (15 m length × 0.32 mm id × 1.0 μm film thickness). 1.0 μL of sample solution was injected into the split port of the GC apparatus maintained at 150 °C using a constant pressure of 4.79 psig. The split ratio was set at 13.9:1 resulting in a split flow of 48.0 mL/min and a column flow of 3.6 mL/min (54.1 mL/min total flow). The carrier gas used was helium. The gradient oven program started at an initial temperature of 80 °C, increased at 20 °C/min to 150 °C which was held for 2 min, then increased at 10 °C/min to 250 °C, then immediately increased at 40 °C/min to 300 °C and held for 2 min.

The MSD used a source temperature set at 230 °C and a quadrupole temperature of 150 °C. Emission was set at 34.6 μA and electron energy at 69.9 eV. The transfer line temperature was set at 310 °C. Data acquisition began after a 4 min solvent delay to preserve the MS filaments. Acquisition mode was SIM at *m*/*z* 168. The SIM chromatograms for a blank, a 0.2 ppm derivatized HOPO standard and a API sample are shown overlaid in Fig. 6. The SIM chromatograms for API spiked with 0.2 ppm HOPO and unspiked API sample are shown overlaid in Fig. 7.

3.6. Analysis criteria

The working standard solution is injected to bracket the API samples. The system suitability criterion for % Relative Standard



Key:
1 - API Spiked with 0.2 ppm HOPO
2 - Unspiked API
* - Derivatized HOPO peak at retention time 5.9 minutes.

Fig. 7. SIM overlay of API spiked with 0.2 ppm HOPO and unspiked API.

Deviation (%RSD) of the HOPO peak area from six injections of the working standard solution was set at ≤10.0%.

The HOPO content (ppm) of the API sample was calculated using the following formula:

$$\frac{A_{samp}}{A_{std}} \times C_{std} \times \frac{V_{samp}}{W_{samp}} \times 1000000$$

In which C_{std} is the concentration (mg/mL) of HOPO in the working standard; V_{samp} is the total volume (mL) of the API sample preparation (determined to be 1.17 mL); W_{samp} is the sample weight (mg); A_{samp} and A_{std} are the peak area responses obtained from the API sample and working standard preparations, respectively.

3.7. Method validation

Method validation procedures were carried out in accordance with ICH guidelines [10]. The specificity of the method was demonstrated by assessing peak area interference at the m/z retention time of HOPO using SIM. Linearity of the derivatized HOPO peak response was determined across a concentration span of 0.2–5 ppm using seven different concentrations. Least squares regression indicated that over this range, the data fits a linear model, with a y-intercept not significantly different from zero. The derivatization process was performed and the recovery of the HOPO calculated for API spiked at three concentration levels (0.2, 1.2 and 2.4 ppm) with three preparations at each level to assess accuracy, Table 3. All recoveries demonstrated acceptable accuracy.

Table 3
Spike and recovery of HOPO from API sample solutions ($n = 3$).

% Recovery	Concentration (ppm)		
	0.2	1.2	2.4
1	79.5	104.2	114.1
2	83.5	112.6	120.7
3	85.1	111.0	121.9
Mean	82.7	109.3	118.9
SD	2.9	4.5	4.2
%RSD	3.5	4.1	3.5

Table 4
Sample repeatability ($n = 6$).

Preparation	API Batch 1 (HOPO level (ppm))	API Batch 2 (HOPO level (ppm))
1	12.6	0.6
2	14.9	0.6
3	16.1	0.6
4	16.3	0.6
5	17.5	0.6
6	16.2	0.7
Mean	15.6	0.6
SD	1.69	0.07
%RSD	10.8	11.5

Injection precision was determined by calculating the % Relative Standard Deviation (%RSD) of the peak area for six injections of the working standard solution. Sample precision was determined by calculating the % RSD for the peak area for six preparations of two batches of API, Table 4. Intermediate precision was assessed by analyzing three API samples from three batches on each of three days, Table 5. Sample precision was also repeated by a different analyst on a different day. The %RSDs calculated for both injection and sample repeatability indicated excellent precision.

Table 5
Intermediate precision.

Batch	Prep	HOPO (ppm)		
		Day 1	Day 2	Day 3
1	1	0.6*	0.5	0.5
	2	0.6*	0.6	0.6
	3	0.6*	0.6	0.6
2	1	13.3	12.0	12.8
	2	13.0	11.9	12.4
	3	13.3	12.2	11.5
3	1	12.6	13.0	14.6
	2	14.9	14.4	15.2
	3	16.1	14.7	14.9

* Different analyst.

Table 6
Chromatographic parameters evaluated for robustness.

Parameter	Range
Injector inlet temperature	145–155 °C
Intial oven temperature	78–82 °C
MS source temperature	218–242 °C
MS quadrupole temperature	142–158 °C
Derivatisation time	54–66 min
Derivatiation temperature	54–66 °C
Initial flow rate	3.5–3.7 mL/min
Split flow rate	45–50 mL/min
Column packing material	3 lots

For method sensitivity, the detection limit (DL) and minimum quantitation limit (MQL) are expressed as:

$$DL = \frac{3.3\,SD}{S} \quad MQL = \frac{10\,SD}{S}$$

where: S = slope of the linearity curve; SD = standard deviation of peak area response of six replicate injections at the reporting level (0.2 ppm).

Based on this estimate the DL and MQL were determined to be approximately 0.06 and 0.19 ppm for HOPO relative to the API. These results demonstrate that the sensitivity of the method is appropriate for the determination of residual HOPO at trace levels.

For solution robustness, the stability of the 0.2 ppm standard solution and API sample solutions (in pierced and unpierced sample injection vials) were assessed after 24 h at RT/RL against freshly prepared standards, Table 2. The results indicated the standard and sample solutions are stable for 24 h stored at ambient temperature/room light conditions. There was acceptable change in the ppm level of HOPO in the sample solution after 24 h of storage in pierced or unpierced injection vials.

The ruggedness of the method was tested by making deliberate changes to the parameters as described in Table 6 and monitoring the effect on precision of injection of a working standard solution. For column robustness, the mean retention time (minutes) of the derivatized HOPO peak was also determined and the % difference between columns calculated. Results are shown in Table 7 and demonstrate that the operating thresholds of the method are suitably robust.

Table 7
Ruggedness - method robustness.

Injector inlet temperature (°C)	145	150	155
%RSD of peak area of n = 6 injections	0.4	0.6	0.8
Intial oven temperature (°C)	78	80	82
%RSD of peak area of n = 6 injections	0.4	0.6	0.4
Source temperature (°C)	218	230	242
%RSD of peak area of n = 6 injections	1.0	1.3	1.1
Quadrupole temperature (°C)	142	150	158
%RSD of peak area of n = 6 injections	0.5	1.3	0.6
Derivatization (min)	54	60	66
%RSD of peak area of n = 6 injections	0.7	1.2	0.7
Derivatization temperature ((°C)	54	60	66
%RSD of peak area of n = 6 injections	0.9	1.2	0.3
Initial flow rate (mL/min)	3.5	3.6	3.7
%RSD of peak area of n = 6 injections	2.0	5.1	0.8
Split flow rate (mL/min)	45	48	50
%RSD of peak area of n = 6 injections	1.3	3.3	0.4
Column serial number	1061690	1061689	1070644
%RSD of peak area of n = 6 injections	1.1	0.6	1.2
Mean retention time of n = 6 injections (min)	5.9	5.9	5.9

The method validation parameters evaluated and the results obtained are summarised in Table 8. The validation demonstrated that the method was suitable for use.

Table 8
Method validation summary.

Validation parameter	Procedure	Result
Specificity	Assess interference at the *m/z* retention time (RT) of the analyte using SIM.	No significant interference of the analyte peak was noted.
Linearity	Linearity of derivatised HOPO was evaluated from 0.2 to 5 ppm (range of 16–417% of the working standard concentration) using seven standard concentrations.	R = 0.9999
Accuracy (spike and recovery of HOPO in API samples)	Spike and recovery experiments were performed at three concentration levels (0.2, 1.2 and 2.4 ppm) with three preparations at each level.	Average recovery (n = 3): 0.2 ppm = 82.7% 1.2 ppm = 109.3% 2.4 ppm = 118.9%
Precision:		
Injection repeatability	%RSD of the peak area for six injections of the working standard solution.	%RSD = 1.08
Sample repeatability	%RSD of the peak area for six preparations of one batch of API.	Batch 1: %RSD = 10.8 (n = 6) Batch 2: %RSD = 11.5 (n = 6)
Intermediate precision (reproducibility)	%RSD of three API samples from three batches on each of three days. %RSD of six preparations of one sample by a different analyst on a different day to be ≤ 30%	%RSD: Batch #1 = 7.6 Batch #2 = 5.2 Batch #3 = 7.4 %RSD = 10.8
Sensitivity	The detection limit (DL) and minimum quantitation limit (MQL) relative to the API were calculated.	DL = 0.06 ppm MQL = 0.19 ppm
Robustness: Solution stability	Stability of derivatized standard and sample solutions were assessed after 24 hours storage at RT/RL against freshly prepared standards.	Recovery: Standard 1 = 95.3% Standard 2 = 97.1% Sample 1(unpierced) = 107.4% Standard 2 (pierced) = 110.4%
Ruggedness	Robustness of the method was assessed by making deliberate changes to the parameters as described in Table 6. Six injections of the working standard solution were made and the %RSD determined for each robustness assessment. For column robustness the mean retention time (mins) was also assessed and the % difference between columns calculated.	The criteria were achieved at all conditions, see Table 7.

4. Conclusions

A GCMS method was developed and validated for the quantification of HOPO in API. Derivatization was used to overcome the volatility and separation challenges that were observed with HOPO. The method uses MTBSTFA + 1% t-BDMCS as the derivatizing reagent to form HOPO-tBDMS and uses SIM to quantitate the derivatized HOPO. The method has a QL of 0.19 ppm and was fully validated in accordance with ICH guidelines [10].

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