HPLC–UV Method Development and Validation for the Determination of Low Level Formaldehyde in a Drug Substance

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Abstract

A reversed-phase high-performance liquid chromatographic method (HPLC) with diode-array detection is developed and validated for the quantitative determination of formaldehyde in a drug substance. Formaldehyde (HCHO) is reacted with 2,4-dinitrophenylhydrazine (DNPH) to form a Schiff base (HCHO-DNPH derivatization product), which has an absorbing maximum (λ_{max}) at 360 nm. The HPLC method employs a C8, 3-µm particle size analytical column (150 mm × 4.6 mm), 15-µL injection volume, column temperature controlled at 30°C, detection at 360 nm, and a water–acetonitrile (55:45, v/v) mobile phase at a flow rate of 1 mL/min. These conditions resolve the HCHO–DNPH product from unreacted DNPH, the drug substance and related impurities, as well as diluent peaks within 20 min. The retention time of the HCHO–DNPH product is approximately 6.4 min. The method is linear, accurate in the specified range (0.33–333 ppm), and robust based on analyte (HCHO–DNPH derivatization product) stability in standard and sample. Detection limit is 0.03 ng (0.1 ppm).

Introduction

Regulations require the analysis and control of impurities in drug substances (DS), also called pharmaceutical active ingredient, and in drug products to ensure the identity, strength, quality, and potency of drug products. ICH (International Harmonization Conference) has issued several guidelines on how to classify, qualify, report and control impurities: ICH (2002) Q3A for impurities in new drug substances (1), ICH (2003) Q3B for impurities in new drug products (2), and ICH (1997) Q3C (3), and ICH (1999) Q3C(M) (4) for residual solvents.

For one of our DS during an early stage of development, in the final step of DS synthesis, triphenyl phosphine palladium dichloride was used as a catalyst. Because it is a potential inorganic impurity, palladium has to be controlled. Trikis hydroxymethylene phosphine (THP) prepared in-house from tetrakis hydroxymethylene phosphonium chloride and potassium hydroxide were used for palladium removal. Formaldehyde was generated as a byproduct during this step. Thus, the quantitation and control of formaldehyde in the DS was very important besides other requirements.

Formaldehyde is not on the ICH guideline lists for solvents and thus a control limit cannot be found. Per World Health Organization's guideline WHO/SDE/WSH/05.08/48, formaldehyde is carcinogenic by inhalation but is not carcinogenic by the oral route. In the gastrointestinal tract, formaldehyde is rapidly oxidized to form formic acid, a class 3 solvent per ICH. The US Environmental Protection Agency (EPA) has established a maximum daily dose reference (RfD) of 0.2 mg/kg per day for formaldehyde (5). At exposures increasingly greater than the RfD, the potential for adverse health effects increases. A health-based guideline value of 0.35 mg/L (350 µg/L) for formaldehyde in drinking water is derived.

Considering all these guidelines and taking a conservative approach, our control level of formaldehyde on the basis of a maximum daily dose (2400 mg for its intended purpose) and the 700 µg formaldehyde allowed translates into a concentration of 290 ppm in drug substance. Ideally, the method for formaldehyde would be able to quantitate it below 290 ppm.

Formaldehyde is a small molecule and has one carbon and one heterogeneous oxygen atom. This molecule is not readily amenable to gas chromatographic (GC) with flame ionization detection (FID). Also, formaldehyde is not easily ionizable and cannot be easily analyzed by mass spectrometry (MS). The analysis of formaldehyde is commonly achieved by a high-performance liquid chromatography (HPLC) method following a derivatization reaction with 2,4-dinitrophenylhydrazine (6). The technique has been reported for analyses of cosmetics (7), tap water (8), fish-paste products (9), natural gas and oil combustion products (10), aqueous extracts, and model mixtures simulating foods (11). Hyphenated techniques such as GC–MS and LC–MS–MS (EPA Method 8315) can be used as a confirmatory technique. GC–ECD method (EPA Method 556) uses pentafluorobenzyl hydroxylamine (PFBHA) to form oxime derivatives of aldehydes in an aqueous solution at pH 4. The derivatives are

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extracted into hexane and analyzed by GC–ECD (detection limit approximately < 1 µg/L). For GC–MS, a derivative is extracted from aqueous solution using a suitable solvent such as toluene. Although the GC–MS method has better specificity, the GC–MS method offers a detection limit approximately 10 µg/L, inferior to the HPLC method. LC–MS–MS can also be done. The detection limit is 20 µg/L, because the MS–MS interferences are eliminated. Two reports have been published for the analysis of formaldehyde in pharmaceutical products, enteric coating of hard gelatin capsules (12), and injectibles (13). In this paper, we report a validated HPLC method for analyzing formaldehyde in a DS sample by using the 2,4-dinitrophenylhydrazine (DNPH) derivatization reaction. To our knowledge, this is the first paper for the analysis of formaldehyde in a DS matrix.

Experimental

Chemicals and reagents

OmniSolv HPLC grade acetonitrile (ACN) and phosphoric acid 85% (Wt.) HPLC grade were purchased from EM Science (Gibbstown, NJ). Water was purified from a Milli-Q purification system (Milford, MA). Formaldehyde 37% wt. % solution in water, A.C.S. reagent, was purchased from Sigma Aldrich (St. Louis, MO).

2,4-Dinitrophenyl-hydrazine (DNPH), 97% reagent, was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). Drug substance was prepared and characterized by Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT).

Instrument and conditions

All the analytical studies were performed on an HP series 1100 system (Agilent, Wilmington, DE) driven by ChemStation 8.03 software. This system was composed of a quaternary pump (G1311A), an autosampler (G1329A), a mobile phase degasser (G1322A), and a diode array detector (G1315A).

A reversed phase analytical column, ACE C8 (MAC-MOD Analytical Inc., Chadds Ford, PA) was used. Mobile phase used was water–acetonitrile (55:45, v/v) and detection wavelength at 360 nm. The injection volume was 15 µL and flow rate was set at 1.0 mL/min.

Table I. Linearity of HCHO–DNPH Derivative

HCHO–DNPH derivative conc. (ppm) vs. BI drug substance	Peak area
0.33	2535
1.67	13086
3.33	23822
33.3	231098
100	699064
200	1384088
267	1810330
333	2271662

Slope	*Y*-Intercept	Correlation coefficient (*r*)
6813	5074	0.99988

Solution preparation

0.1 µg/mL of HCHO solution

To a 50-mL volumetric flask was added 124 µL of 37% formaldehyde solution with a piston pipette and diluted to volume with water. This solution was labeled 1 mg/mL HCHO.

A 1 mL aliquot of the 1 mg/mL solution was pipetted into a 100-mL volumetric flask and diluted to volume with water. This solution was labeled 10 µg/mL HCHO.

A 1 mL aliquot of the 10 µg/mL solution was pipetted into a 100-mL volumetric flask and diluted to volume with water.

5N H_3PO_4 solution

Approximately 9.60 ± 0.10 gm of phosphoric acid solution were accurately weighed into a 50-mL volumetric flask with a piston pipette and diluted to volume with water.

1 mg/mL DNPH Solution

Approximately 51.50 ± 0.50 mg of 2,4-DNPH (97%) into a 50-mL were accurately weighed into a volumetric flask (1 mg/mL) and approximately 10 mL of ACN were added to the flask. It was sonicated for 3 min and diluted to volume with ACN.

Kinetics of derivatization experiment

To a 20-mL scintillation vial, 10 mL of formaldehyde solution (0.1 µg/mL), 200 µL of phosphoric acid (5N), and 2 mL of 2,4-dinitrophenylhydrazine reagent (1 mg/mL) were added. The mixture was stirred for 5, 15, 30, 60, 120, 180, and 240 min. At each interval, 1 mL of the mixture was pipetted into a glass vial and diluted with 1.0 mL of acetonitrile. This diluted reaction mixture was then analyzed by HPLC. Formaldehyde was quantitatively converted to the Schiff base in 30 min, which is in accordance with Selim in an analogous study with propionaldehyde (14). All derivatization experiments reported in this manuscript were using the 30 min optimized reaction time.

Standard and sample preparations

Derivatized standard

To a suitable vial, 1 mL of 0.1 µg/mL formaldehyde standard solution, 20 µL of 5N phosphoric acid, and 200 µL of 2,4-dinitrophenylhydrazine solution were added. It was stirred it for at least 30 min and then 1 mL of acetonitrile was added.

Table II. Recovery of HCHO–DNPH Derivative*

Conc. ppm	Preparation #	% Recovery
0.66	1	93.6
	2	100.1
33.3	1	101.3
	2	101.0
333	1	99.8
	2	99.8
	Average (*n* = 6)	99
	Overall RSD (%)	2.9

* Note: Precision was established based on the satisfactory accuracy (RSD ≤ 5.0%).

Drug substance stock solution (30 mg/mL)

Approximately 150.00 ± 0.5 mg of drug substance sample was accurately weighed and transferred to a 5-mL volumetric flask. Four mL of ACN were added and sonicated for 8 min. It was diluted to volume with ACN.

Derivatized sample

To a suitable vial, 1 mL of 30 mg/mL drug substance stock solution, 20 µL of 5N phosphoric acid, and 200 µL of 2,4-dinitrophenylhydrazine solution were added. It was stirred for at least 30 min and then 1 mL of water was added.

Results and Discussion

Method development

The HPLC impurity method for the drug substance was used as an initial platform for the analysis of formaldehyde. The ACE C8, 150 mm × 4.6 mm HPLC column was used. Mobile phase was (50:50, v/v) acetonitrile (B) and water (A). When detected at 254 nm, peaks from the DS were detected posing strong interference. When detected at 360 nm (Figure 1), the sensitivity of the HCHO–DNPH derivatization product and unreacted DNPH was dramatically improved, whereas the interference of the DS and related impurities as well as diluent components was suppressed to the negligible level.

Reducing the mobile phase ratio to 45:55 (v/v, B/A) yielded the optimal separation (see Figure 2).

Method Validation

The procedure is intended to be used as a limit test to monitor formaldehyde in drug substance. Specificity, linearity, accuracy, precision, QL, DL, and solution stability were also established.

Specificity

Specificity was demonstrated by injecting diluent, drug substance control sample (15 mg/mL), reagent blank, DNPH, 5N phosphoric acid, and formaldehyde solution (10 µg/mL) individually. Figure 2 shows overlays of chromatograms for diluent background, HCHO, DNPH, underivatized sample, and derivatization standard, and derivatized sample. No interference with the HCHO–DNPH derivatization product was observed for the drug substance investigated. The retention times of DNPH and the HCHO–DNPH derivatization product were 3.8 and 6.4 min, respectively. The peaks in the derivatized sample were identified by comparing retention times and UV–vis spectra with those of peaks in DNPH and the derivatized standard. Furthermore, the integrity of the HCHO–DNPH peak was confirmed by overlaying UV spectra from 7 positions of the peak and by algorithmic analysis of spectra. Clearly, the method is selective for the analysis of formaldehyde in the given DS sample matrix.

Table III. Analysis of Formaldehyde in BI Drug Substance*

Drug substance	HCHO vs. drug substance (ppm)
1	ND (< DL)
2	0.4
3	0.3 (< QL)

* ND = Not detected, QL = 0.33 ppm, DL = 0.13 ppm.

Linearity

Calibration curve

Six standard solutions of formaldehyde 2,4-dinitrophenylhydrazone (0.33–333 ppm, which corresponds to 0.1–100% of the standard preparation concentration) were prepared according to Table I. A 15 µL solution was then injected in the chromatograph and a calibration curve was obtained by linear regression of the peak-area of formaldehyde 2,4-dintrophenylhydrazone versus concentrations.

The regression equation obtained was: $Y = 6813X + 5074$, where Y is peak area of the derivatization product and X is free formaldehyde concentration in ppm. The correlation coefficient of 0.99994 ($n = 8$) proved excellent linearity between derivatization product peak area and free formaldehyde concentration in the concentration range of 0.33–333 ppm. Formaldehyde concentrations were determined from the regression equation. Results for these experiments are summarized in Table I.

Accuracy (Recovery)

The accuracy of the procedure was demonstrated by the recovery studies, which were carried out by spiking aliquots of drug substance stock solution with formaldehyde at three levels corresponding to 0.66 ppm, 33.3 ppm, and 333 ppm. Because the drug substance is in the early stage development (phase I) and the method is used as a limit test in-process testing, full accuracy

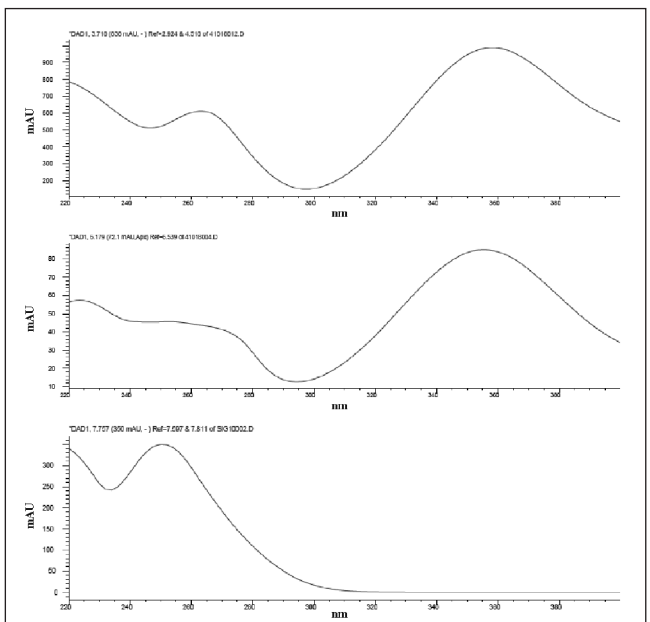

Figure 1. UV spectra at peak apex. DNPH (0.5 mg/mL) (A); HCHO–DNPH (1.5 µg/mL); drug substance (1 mg/mL).

validation per ICH was not performed. Rather, two separate preparations were made at each level and each preparation was injected once. The average recovery was 99% with a relative standard deviation (RSD) of 2.9%, indicating a very high accuracy. Results are summarized in Table II.

Detection limit (DL) and quantitation limit (QL)

The 0.33 ppm test concentration peak of DNPH-Formaldehyde was injected twice, and a signal-to-noise ratio of 13 was obtained. Thus the method QL and DL were set at 0.33 ppm (~0.1 ng) and 0.10 ppm (~0.03 ng). Figure 3 shows the chromatogram at the QL level. If based on formaldehyde itself (not with respect to DS), QL was found to be 5 ppb, and DL is 2 ppb.

In comparison, reference 15 reported a formaldehyde DL of 1.1 ng for analyzing air samples by using the same derivatization reaction and detection technique. DL of approximately 4 ng (16) was reported for analyzing several lots of a penicillin-type antibiotic tested for possible formaldehyde contamination using hydroxylamine hydrochloride as a derivatization reagent and detection using GC equipped with nitrogen-specific detector. Also, several lots of protein concentrate using chromotopic acid spectrophotometric analysis to detect trace level of formaldehyde afforded DL of 100 ng (16).

It is fair to compare sensitivity by different methods based on formaldehyde itself, as sensitivity is primarily affected by a derivatization reaction, detection technique (UV or MS), or chromatographic conditions (e.g., HPLC vs GC, column dimensions, efficiency). If sensitivity (in terms of DL) is based on sample (e.g., vs. drug substance), then sensitivity is additionally affected by the choice of the derivatization solvent, sample solubility, matrix interference, etc. In this paper, the drug substance (DS) happens to have a high solubility (30 mg/mL) in the solvent chosen, thus QL (0.33 ppm) and DL (0.1 ppm) are enhanced.

Stability of analyte solutions

The solution stability of the standard (3.33 ppm) and the representative drug substance sample spiked at 3.33 ppm with formaldehyde was monitored by comparing assay values over a period of 48 h against a freshly prepared standard. Both standard and sample solutions were found stable for at least 48 h at ambient condition, since assay values varied within 2% of the initial value.

Sample analysis

Three lots of drug substance were assessed for their formaldehyde content. Results of the amount of formaldehyde in these lots (summarized in Table III) indicated that the purification procedure was effective to remove residual formaldehyde in the DS or at least keep it down to trace levels.

Conclusions

This report presents the development and validation of a simple isocratic HPLC procedure suitable for the analysis of formaldehyde in a drug substance. It was demonstrated that the procedure developed is sensitive, accurate, precise, and robust (analyte stability). This derivatization (using 2,4-dinitrophenylhydrazine) reaction and detection at 360 nm are expected to be applicable to analysis of formaldehyde in other drug substance samples as long as the drug substance is soluble in the derivatization solvent chosen and the drug substance does not absorb appreciably above 300 nm.

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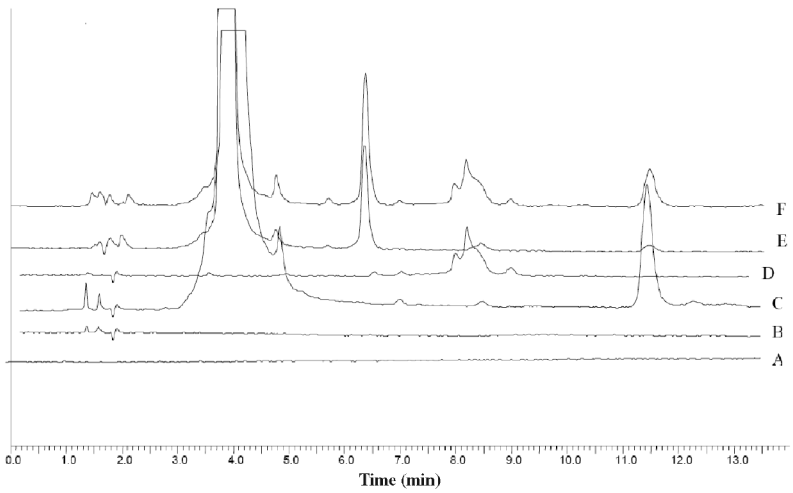

Figure 2. Selectivity chromatograms (overlays): mobile phase (blank) (A); HCHO (blank) (B); DNPH (C); underivatized BI drug substance sample (D); derivitized standard (final conc. of formaldehyde as HCHO–DNPH is ~ 3.33 ppm) (E); derivatization sample (sample spiked with formaldehyde, final conc. of HCHO–DNPH is ~ 3.33 ppm) (F). Substance and retention time (min): DNPH, 3.82; HCHO–DNPH, 6.37; BI drug substance, 8.14.

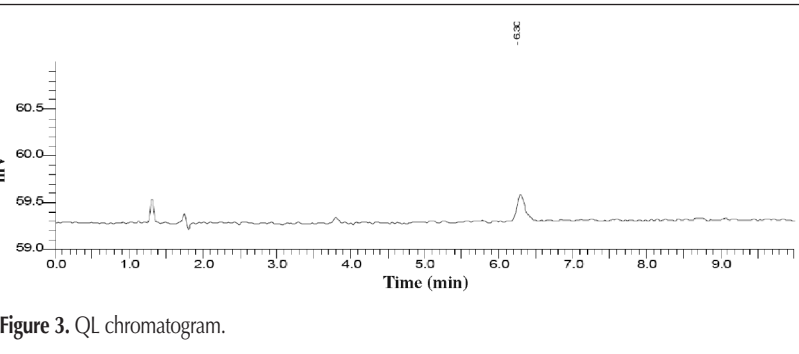

Figure 3. QL chromatogram.

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