Low Level Determination of 4-Amino-2-Ethoxy-Cinnamic Acid and its Ethyl Ester in a Drug Substance and its Formulation Prototypes by HPLC–UV-DAD

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Abstract

A reversed-phase high-performance liquid chromatographic method (HPLC) with diode-array detection (DAD) has been evaluated for monitoring trace levels of impurities, such as 4-amino-2-ethoxy-cinnamic acid (impurity A), hydrochloride salt of 4-amino-2-ethoxy-ethyl cinnamate (impurity B), and 4-bromo-3-ethoxy-nitrobenzene (impurity C), in drug substance and 3 different formulation prototypes. These compounds have been highlighted as potential genotoxins and 2-ethoxy-4-amino-cinnamic acid (impurity A) as possible degradant isolated during the synthesis of BI drug substance. HPLC–UV-DAD was found to be more promising, and limits of quantification were between 0.09 and 0.6 µg/mL, which enabled detection limits in drug substance at 2–15 ppm for a 15 mg/mL solution. All three genotoxic impurities are completely resolved from each other as well as from diluent peaks, drug substance, and other related impurities within 40 min. The retention times of impurities A, B, and C were 3.4, 13.1, and 21.3 min. The results demonstrating the specificity, assay precision, recovery, linearity, and range achieved during the method validation experiments are presented in this paper.

Introduction

Residual impurities resulting from manufacturing and formulation, or from degradation of the active pharmaceutical ingredient (API) and excipients, may be present in pharmaceutical products. A subset of these impurities (reactive intermediates used in the synthetic route for the production of API) may present a potential for genotoxicity and therefore pose an additional safety concern to clinical subjects and patients. Therefore, substantial efforts are made during development to control all impurities at safe concentrations.

The presence of low-level genotoxic impurities in pharmaceutical products has been documented (1–4). These are often chemical reagents used in the synthetic process (5) or low-level contaminants generated during synthesis (6). The pharmaceutical industry recognizes the importance of limiting exposure to genotoxic impurities and takes measures to control these impurities during development. However, low levels of impurities with genotoxic potential may be unavoidable in some cases, especially during early phases of clinical development.

Formal approaches to manage both the quality of API and safety risk of drug impurities have been reviewed and discussed among pharmaceutical industry sponsors and regulators (7). Guidelines have been introduced through the International Conference on Harmonization (ICH) process, which incorporate synthesis, manufacturing, analytical, toxicological, and regulatory considerations for managing impurities. With the exception of ICH Q3C recommendations for the control of some carcinogenic solvents, these guidelines do not address genotoxic impurities specifically, although it is suggested that such impurities should be controlled more stringently. Toxicological assessment and justifications of limits per these ICH guidelines are normally based on the qualification of representative batches of the API including its impurities in pivotal toxicity studies that include genetic toxicology tests.

Acknowledging this, the European Medicines Agency Committee for Medicinal Products for Human Use (CHMP) has issued guidance for the control of genotoxic impurities in new drug products (8). The CHMP has issued a Draft Guideline on the Limits of Genotoxic Impurities, which describes an approach for assessing genotoxic impurities of unknown carcinogenic potential or potency based on the Threshold of Toxicological Concern (TTC) concept (9). The extension of CHMP approach includes the concept of a staged TTC that establishes allowable daily intakes of impurities based upon duration of exposure.

Recently, a Genotoxic Impurities Task Force consisting of experts from the pharmaceutical industry has recommended a process for testing, classifying and controlling genotoxic impurities during all stages of development (10). Process intermediates and potential impurities are classified based on a structural assessment of mutagenic and carcinogenic potential and are controlled or managed accordingly.

Aniline derivatives such as dimethyl, trimeyhyl, and p-amino cinnamic acid are often used during manufacture of pharmaceuticals. In fact, alkyl substituted anilines are known genotoxins and are known carcinogens in rats and mice (11). The potential presence of these genotoxins has attracted the attention of regulatory authorities. Because these impurities have no therapeutic benefit to the patient population or healthy subjects enrolling in clinical studies, it is preferable for the potential genotoxins to be controlled during the synthesis. In cases where levels cannot be controlled and no safety data exists, it may be preferable for the

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pharmaceutical company to change the route of the drug substance isolation procedure, though this normally happens only during early development.

As BI had one drug in early stage development using 4-amino-2-ethoxy-ethyl cinnamate (impurity B) as one of the key intermediates, it was felt necessary to develop a simple, sensitive, and validated method. In the early stage development of drug substance (DS), control of these impurities at low levels (< 20 ppm) is of prime importance, as the final dose is not always known. It was also important that the methodology could easily be adapted to other APIs or intermediates, ideally with direct injection onto a column, avoiding tedious sample work-up.

The structures of these three potential genotoxic impurities (based on structure activity relationship and later on confirmed by Ames test) present in BI drug substance (early development candidate) are listed in Figure 1.

Figure 1A is identified as potential metabolite and also formed as a degradation product under acidic conditions. Figure 1B is one of the key intermediates used in synthesis of the intermediate, which is an immediate precursor to BI drug substance (isolated two steps upstream from the final step). Figure 1C is the starting material used to synthesize impurity B.

Traditionally, for determination of nitrobenzene in environmental and biological samples, high resolution gas chromatography (GC)–flame ionization detection (12) is routinely used. Also, earlier publications have described methods for the analysis of cinnamic acid and its derivatives from methanolic and aqueous plant extracts using GC–MS (13) and HPLC (14,15) methods. The detection limits of cinnamic acid derivatives for the described GC–MS (SIM) method of analysis ranged between 2 and 40 ng/mL, whereas limits of quantitation fell in the range of 5–118 ng/mL. To reach such low levels without sample cleanup (solid-phase extraction), derivatization, and use of hyphenated techniques such as LC–MS or GC–MS is not always possible. To our knowledge, this is the first paper for the analysis of these compounds in a DS matrix.

This short paper describes a very brief evaluation of sensitive direct injection HPLC–UV-DAD approach, which has been used for the drug substance (both as salt and free acid), to support process optimization and formulation development efforts, and has been found applicable to other pharmaceuticals under development.

Experimental

Chemicals and reagents

Acetonitrile (ACN) and methanol, OmniSolv grade, were purchased from EMD (Gibbstown, NJ). Water was purified using a Milli-Q purification system (Bedford, MA). Trifluoroacetic acid (TFA), 99.9% reagent, was purchased from Pierce Chemical Company Inc. (Milwaukee, WI). HPLC purities of drug substance (0.3% water content by Karl Fischer), relevant intermediates, and genotoxic impurities (impurites A–C) were obtained from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT) were > 98%.

Instrumentation and operating conditions

All the analytical studies were performed on a 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 (DAD) (G1315A).

Two different HPLC C_{18} columns from Waters (XTerra and Atlantis) were compared initially. The separation of analytes was finally accomplished using an Atlantis dC_{18} reversed-phase column (Waters Corporation Part No.186001342, 4.6 mm i.d. × 150 mm, 3 µm particles). Final chromatographic conditions involved a gradient elution, with solvent A: 0.1% (v/v) TFA in 95% (v/v) water in methanol and solvent B: 0.1% (v/v) TFA in 95% (v/v) acetonitrile in methanol. The gradient was initial isocratic hold time at 20% B for 4 min, followed by linear gradient from 20% to 50% B in 10 min, 50% to 57% in 6 min, 57 to 100% B in 5 min, and a re-equilibration period of 8 min. The column was thermostatted at 35°C, injection volume was 15 µL, and the flow rate was set at 1.5 mL/min. The optimum wavelength of 275 nm, which represents the wavelength of maximum absorbance of all three impurities, was selected in order to permit their simultaneous determination in BI drug substance and different formulation prototypes.

Standard and sample preparations

A stock mixture (0.1 mg/mL) of all impurities was prepared in methanol. Further dilutions were carried to obtain a standard solution of 0.6 µg/mL in DMSO–methanol (1:1).

Drug substance

The test samples (drug substance) were prepared by dissolving 30 mg of the drug substance in 1 mL of DMSO in a 4-mL amber vial, sonicating for 5 min, and adding 1.0 mL of methanol.

Formulation prototype BI drug: PVP–TRIS–SDS (1:1:0.35:0.80)

The test sample was prepared by dissolving 102 mg of formulation (equivalent to 30 mg of active) in 1 mL of DMSO in a 4-mL amber vial, sonicating for 5 min to dissolve, and adding 1.0 mL of methanol.

Formulation prototype BI drug: PVP–TRIS (1:1:0.35)

The test sample was prepared by dissolving 62 mg of formulation (equivalent to 30 mg of active) in 1 mL of DMSO in a 4-mL amber vial, sonicating for 5 min to dissolve, and adding 1.0 mL of methanol.

Formulation prototype BI drug: PVP–TRIS–SDS (1:1)

The test sample was prepared by dissolving 76 mg of formulation (equivalent to 30 mg of active) in 1 mL of DMSO in a 4-mL amber vial, sonicating for 10 min and adding 1.0 mL of methanol. The solution, being cloudy, was filtered through an Acrodisc LC13 (0.45 µm) filter.

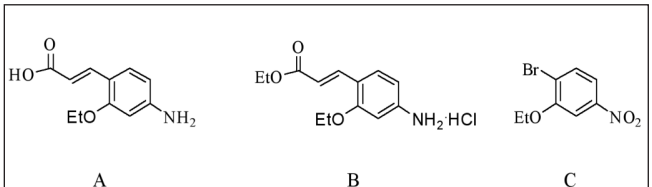

Figure 1. Structures of potential genotoxic impurities (A, B, and C).

Standard and sample preparation for dissolution in SGF with pepsin media at 50 rpm (pH 1.2) at 37°C

A stock mixture (0.1 mg/mL) of all impurities prepared in methanol was further diluted with SGF pepsin media to obtain a standard solution of 0.6 µg/mL. Dilution in media was performed to simulate the sample preparation.

50 mg of dispersion BI drug: PVP–TRIS–SDS (300 mg:300 mg:200 mg:200 mg) (equivalent to 15 mg active) in SGF with pepsin media (pH 1.2) and samples were pulled after 1 h and 2 h.

Results and Discussion

Method development

The HPLC–UV-DAD impurity method for the drug substance was used as an initial platform for the analysis of impurities A, B, and C. The Waters XTerra RP_{18}, 100 × 4.6 mm HPLC column was used. Eluent A was 0.1% (v/v) TFA in 95% (v/v) water in methanol and Eluent B was 95% (v/v) acetonitrile in methanol. The gradient was kept the same as mentioned earlier in the "Instrumentation and operating conditions" section.

When detected at 275 nm, substance A (Figure 1) did not show adequate retention on this column, and peak from the diluent (1:1) DMSO–methanol was detected posing strong interference with impurity A. By changing the column to silica-based line of difunctionally bonded reversed-phase Atlantis dC_{18} column, which exhibits superior retention of polar compounds, the interfering peak from diluent was well resolved from impurity A.

Method validation

The procedure is intended to be used as a limit test to monitor genotoxic impurities in drug substance. Specificity, linearity, accuracy, precision, quantitation and detection limits (QL and DL), and solution stability were also established.

Specificity

The specificity was evaluated by individual injection of three reference standards, the diluent blank, the working standard solution mixture, selectivity mix solution (potential degradation products/intermediates isolated during synthesis), and unspiked (control) and spiked BI drug substance sample (15 mg/mL). In regard to specificity, there was evidence that the substances being quantitated were the intended analytes.

No interference was at the same or at ± 5% of retention time of each known impurity when the analytes were individually analyzed, and all of the impurities were well resolved from the drug. In addition, some other unknown compounds present in the sample of drug substance were also resolved (Figure 2). Furthermore, the integrity of the each impurity peak was confirmed by overlaying UV spectra from 7 positions of the peak and by algorithmic analysis of spectra (Figure 3).

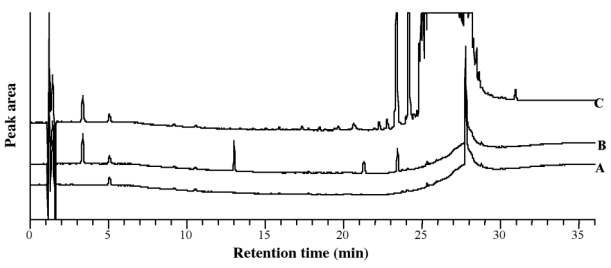

Figure 2. Selectivity chromatograms (overlays): A = diluent (blank); B = 40 ppm standard (final conc. of each impurity peak is ~0.6 µg/mL); C = BI drug substance sample (Lot 2) (conc. 15 mg/mL). 4-Amino-2-ethoxy-cinnamic acid (RT = 3.4); hydrochloride salt of 4-amino-2-ethoxy-ethyl cinnamate (RT = 13.1); 4-bromo-3-ethoxy-nitrobenzene (RT = 21.3). Where RT is approximate retention time in minutes (in overlay B for 40 ppm standard).

Linearity

The linearity of impurity A and hydrochloride salt of impurity B were satisfactorily demonstrated with a eight point calibration graph between 0.09 and 6.0 µg/mL (6–400 ppm with respect to a sample concentration of 15 mg/mL). Also, linearity of di-substituted nitrobenzene (impurity C, Figure 1C) was satisfactorily demonstrated with a five point calibration graph between 0.30 and 6.0 µg/mL (20–400 ppm with respect to a sample concentration of 15 mg/mL). Correlation coefficients for all analytes were > 0.9995.

Accuracy (recovery)

The accuracy of the method was verified by the analysis of spiked drug substance samples in the range of ~ 0.3 to 0.75 µg/mL (20 to 50 ppm based on sample conc. of 15 mg/mL) of impurities. Three separate preparations were made at each level, and each preparation was injected once. The drug substance sample that was not spiked was injected once. For a given impurity, if there was interference from the unspiked sample, the peak area of the unspiked sample injection was subtracted from that of the spiked sample injection.

Results are summarized in Table I. The results show that the method is able to accurately quantitate the impurities within the studied range.

Precision

Six preparations from the same lot were analyzed. Duplicate

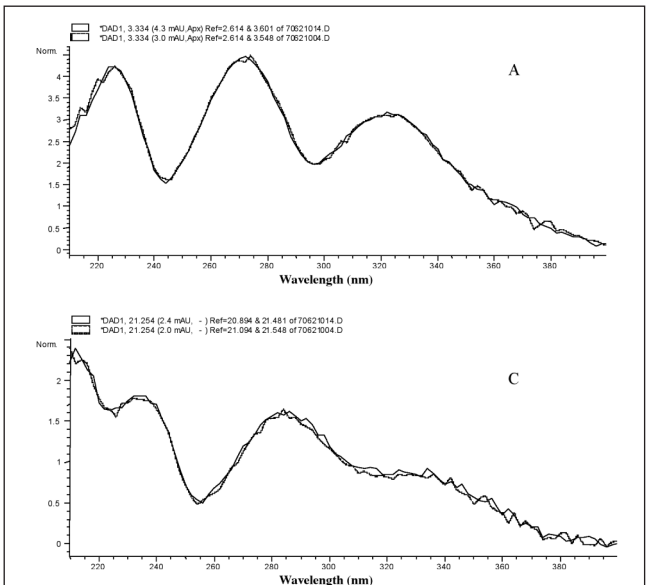

Figure 3. UV spectra at peak apex in sample (Lot 2) and 40 ppm standard (overlays).

injections were made for each preparation. The assay precision, from the results shown in Tables I and II, was less than 5% RSD.

Quantitation and detection limits

The QL was estimated by using the signal to noise ratio (S/N) of the impurities in duplicate injections of 6 to 20 ppm level linearity solution.

The S/N for the 6 ppm solution ranged from 12 to 15 for impurities A and B. The QL was conservatively estimated for an S/N of 10 to be 6 ppm relative to the active. The DL is estimated as one third of the QL level or 2 ppm of the active.

The S/N for the 20 ppm solution ranged from 15 to 17 for compound C. The QL was estimated for a S/N of 10 to be 15 ppm relative to the active. The DL is estimated as one third of the QL level or 5 ppm of the active. The results indicate that the method was sensitive for the intended purpose.

Table I. Recovery (%) from the Accuracy Experiments for the BI Drug Substance*

Level	Preparation #	A	B	C
20 ppm	1	89.6	98.6	98.7
	2	88.5	98.6	95.2
	3	90.1	96.8	95.1
	Mean (± SD)	89.4 (± 0.5)	98.6 (± 0.6)	96.3 (± 1.2)
40 ppm	1	93.5	95.4	88.5
	2	86.7	93.7	87.8
	3	93.1	92.3	90.6
	Mean (± SD)	91.1 (± 2.2)	93.8 (± 0.9)	89.0 (± 0.8)
50 ppm	1	86.1	92.9	95.1
	2	87.4	91.3	93.3
	3	83.4	89.0	91.5
	Mean (± SD)	85.6 (± 1.2)	91.1 (± 1.1)	93.3 (± 1.0)
Overall Mean (± SD)		89. (± 1.1)	94. (± 1.1)	93. (± 1.2)
% RSD (n = 9)		3.7	3.2	3.2

* Note: as the amount spiked is much lower than amount present in the actual sample for A, recovery at QL level cannot be accurately determined, hence is not reported.

Table II. Precision*

Preparation	A (ppm)
1	36
2	36
3	37
4	36
5	35
6	35
Mean (± SD)	36 (± 0.3)
% RSD (*n* = 6)	2.0

* Note: As impurities B and C were not detected in sample, precision for compounds B and C were established based on the satisfactory accuracy (RSD ≤ 5.0%) for nine preparations over 3 levels.

Table III. Solution Stability

Sample ID	Duration	A* (ppm)	B* (ppm)	C† (ppm)
Drug substance (SE) Lot 2	Initial	60	ND‡	50
	5 days	60	ND	47
20 ppm Recovery spiked	Initial	50	18	20
	5 days	48	19	18

* QL = 6 ppm, DL = 2 ppm; † QL = 15 ppm, DL = 5 ppm; ‡ ND = Not detected.

Stability of analyte solutions

The solution stability of the aged standard (40 ppm) preparation was monitored by comparing average calibration factor of peaks of interest over a period of 7 days against freshly prepared standard. The calibration factor (CF) for each component in standard solution is calculated as follows.

$$CF = PAs / Ws$$

Where PAs is the peak area of peak of interest in the standard chromatogram and Ws is the weight of standard in milligrams

Average calibration factor for a particular component is the average of the calibration factor for that component from all standard injections.

The percent change of average calibration factor of freshly prepared standard from initial was not more than 1.1%, 1.3%, and 2.5%, respectively, for impurities A, B, and C. The solution stability of the representative spiked sample and unspiked sample preparations were determined by comparing the levels detected initially against those determined using freshly prepared standard over a period of 5 days. The ppm levels detected after 5 days as compared to initial are summarized in Table III.

Sample analysis

For the purpose of verification, three drug substance batches whose purification and crystallization during the synthesis involved the use of different solvents were analyzed according to this procedure. Two sample preparations for each Lot and one injection per preparation were made. The average results for each batch are shown in Table IV.

During development, to increase solubility, crystalline BI drug (free acid SE form) was converted to its partially crystalline potassium salt form (KA). Numerous lots of these partially crystalline potassium salts (KA) and crystalline free acid (SE) of

Table IV. Analysis of Genotoxic Impurities (ppm) in BI Drug Substance Free Acid (SE)

Drug substance (SE) Lot	A* (ppm)	B* (ppm)	C† (ppm)
1	36	ND	ND
2	60	ND	50
3	449	ND	ND

* QL =6 ppm, DL =2 ppm; † QL =15 ppm, DL =5 ppm; ‡ ND = Not detected.

Table V. Analysis of Genotoxic Impurities (ppm) in BI Drug Substance Salt Form (KA)

Drug substance (KA) Lot	A (ppm)	B (ppm)	C (ppm)
1	362	ND	ND
2	13	ND	50
3	17	ND	ND
4	94	ND	ND
5	17	ND	ND

Table VI. Analysis of Genotoxic Impurities in BI Drug Substance Put on Long Term Storage

A: BI drug (SE, crystalline) Lot 3

	40°C/75%RH, closed		25°C/60%RH, closed
Time zero	4 weeks	3 months	4 weeks
17*	42*	95*	22*

B: BI drug (KA, partially crystalline) Lot 5

	40°C/75%RH, closed			25°C/60%RH, closed
Time zero	4 weeks	3 months	6 months	3 months
16*	16*	25*	29*	17*

* Conc. of A (ppm) detected.

Table VII. Analysis of Genotoxic Impurities in Different Prototypes of BI Drug Substance put on Stability*

Formulation	40°C/75%RH, closed		25°C/60%RH, closed
	Time zero	4 weeks	4 weeks
BI drug: PVP–TRIS–SDS (1:1:0.35:0.80)	229	206	229
BI drug: PVP: TRIS (1:1:0.35)	261	254	290
BI drug: PVP (1:1)	287	266	293
BI drug (Control) ppm	285		

* Conc. of A (ppm) detected.

BI drug substance were tested. Results are shown in Table V.

Representative Lots 3 and 5 were also analyzed for long term stability. Results are shown in Table VI.

Also, three prototype amorphous dispersions, identified based on in vitro dissolution, were also put on stability and monitored for formation of genotoxic impurities, results of which are summarized in Table VII.

The method was also used to get early reads on Ames positive degradant (Impurity A) generation in simulated gastric fluid (from zero to 400 ppm in 1 h and 900 ppm in 2 h) and in a spray-drying process. Based on the results, formulators could get a good idea of controls to use later in the process to avoid its formation.

Conclusion

This method has been demonstrated to be sensitive (DL between 2–5 ppm), specific, accurate, precise, and robust (analyte stability). No matrix-related interference, column overloading, and/or carry over problems were encountered after 12 subsequent sample injections. Because the methodology has none of the restrictions of extraction techniques in which aqueous sample solution must be used, this approach is suitable for both APIs and intermediates that are both soluble in aqueous solutions and organic solvents. This direct injection approach makes it likely applicable to analyzing genotoxic impurities in other drug samples as long as the DS has very high aqueous/organic solubility.

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