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Identification and control of metal-chelating chromatographic artifacts in the analysis of a malonohydrazide derivative drug compound

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ABSTRACT

Two unusual chromatographic artifact peaks were detected in the HPLC analysis for content of a malonohydrazide derivative drug and drug-related impurities. The artifacts were identified as the copper(II) chelating complexes with the drug compound and one of the process impurities. Our investigations suggested that built-up of Cu^{2+} contamination in the HPLC system was the primary source for formation of the chelating artifacts. A rinse procedure using diluted EDTA solution was developed, and demonstrated to effectively purge trace level of heavy metals including Cu^{2+} from the system, and therefore inhibited the formation of both chelates. Furthermore, the rinse was shown to introduce no detrimental impact on the response accuracy of the active drug compound and related impurities.

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1. Introduction

Impurity analyses of pharmaceutical samples often need to use high resolution, high sensitivity, gradient HPLC methods due to regulatory requirements for reporting low levels of drug impurities and complexity of impurity profiles [1,2]. One of the drawbacks associated with HPLC procedure is chromatographic artifacts. Artifact peaks in a chromatogram may interfere with accurate analyses of impurities and the active(s) in a sample and thus compromise the quality and reliability of an HPLC method. When chromatographic peaks of suspect are observed, they need to be investigated and source understood. As a result, product release could be delayed or even worse, a batch could fail the established quality specification because artifact peaks are mistakenly considered as indigenous impurities or contaminants in the concerned batch.

Several examples of artifact peaks and the control to remove them have been described in a review article [3]. The control, ranging from selection of proper quality of mobile phase components, to assurance of cleanliness of glassware and HPLC system can effectively eliminate most common artifact peaks due to trace level of contaminants in the mobile phase, or contaminated glassware. However, identification and control of artifacts induced by other mechanisms are more involved and can become challenging. Sometimes, artifact peaks can be badly reproducible and hardly predictable because they were caused by chemical degradations or unexpected reactions in the sample solutions [4,5]. Occasionally, leachables from the septum of HPLC vial can result in chromatographic artifacts [6]. Another mechanism of artifacts formation is by on-column degradation [7]. Such artifacts can potentially be erroneously labeled as the indigenous impurities inherent to the samples.

In this study, we report identification of a chromatographic artifact peak that resulted from Cu(II) complexation with the active during chromatographic analysis of a malonohydrazide derivative drug compound (a.k.a. GSK842879A). We discovered that one of the drug-related impurities was completely sequestered by Cu^{2+}. An EDTA rinse procedure was developed to prime the HPLC system, which effectively removed built-up of trace level of Cu^{2+} and thus prohibited formation of both artifact peaks.

2. Experimental

2.1. Chemicals

HPLC grade water and acetonitrile were purchased from Fisher Scientific or EMD. Trifluoroacetic acid (spectrophotometric grade) and EDTA di-sodium, dihydrate (ultrapure, bioreagent grade) were obtained from JT Baker. Cu^{2+} solutions were prepared using either copper sulfate, pentahydrate (ACS grade, Sigma–Aldrich), or copper chloride (ACS grade, Aldrich). Fe^{3+}, Co^{2+}, Ni^{2+}, and Zn^{2+} solutions

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(1000 ppm, spectroscopic grade) were purchased from SCP Science.

2.2. HPLC analysis

HPLC analyses were performed on an Agilent HP1100 system equipped with a quaternary pump. A Zorbax SB C18 (150 mm × 4.6 mm, 5 μm) column was thermostated at 30 °C. Mobile phase flow rate was 2.0 mL/min with binary mixing of mobile phase A of 0.005% TFA in water and mobile phase B of 0.005% TFA in acetonitrile. Percentage of mobile phase B was kept at 12% for 10 min from the start and then linearly increased to 70% in the next 20 min followed by a second gradient to 90% in 5 min. Detection wavelength was set at 230 nm, and spectral data acquired from 200 to 600 nm when needed.

2.3. LC–MS analysis

The exact mass measurements were performed using a Q-TOF Premier quadrupole orthogonal acceleration time-of-flight mass spectrometer with LockSpray™ (Waters Corporation, Manchester, UK) controlled by MassLynx 4.1. The electrospray ionization (ESI) source was operated in the positive ion mode. The measured mass was corrected by the internal reference ion with m/z 556.2771 (protonated leucine-enkephalin) which was introduced by the Lockspray.

The MS/MS and H/D exchange experiments were performed with a 2D linear ion trap LTQ mass spectrometer (Thermo Electron, San Jose, CA, USA) controlled by Xcalibur 1.4 software. The ESI source was operated in the positive ion mode. For the H/D exchange experiments, D_2O was substituted for H_2O as mobile phase A.

2.4. Infrared analysis

A Smiths Detection (Danbury, CT) Fourier transform infrared (FT-IR) spectrometer equipped with a diamond attenuated total reflection (DATR) sampling interface was used to measure IR spectra. No sample preparation was performed prior to spectra collection other than applying pressure to ensure good contact between the sample and the optic window. IR spectral data were acquired from 4000 to 400 cm^{-1} with 4 cm^{-1} resolution and 128 co-added scans.

2.5. Study of GSK842879A chelating with selected transition metal ions

GSK842879A solutions were spiked, separately, with Fe^{3+}, Co^{2+}, Ni^{2+}, and Zn^{2+}. The concentration of GSK842879A was 0.2 mg/mL and the metal ion concentrations were 0.01 mg/mL. All solutions were chromatographed on an HPLC system that was fully rinsed with 5 mM EDTA solution to ensure no contamination of transition metals.

2.6. NMR analysis

^1H NMR analysis was conducted on a Varian 400 MHz NMR spectrometer. Sample was prepared in CD_3OD, or 60:40 (v/v) of CD_3CN/D_2O (deuterated solvents were from Cambridge Isotope Labs).

3. Results and discussion

Fig. 1(a) shows a typical chromatogram of a GSK842879A sample solution. The peak at a relative retention time (RRT) of 1.16 (25.0 min) was almost always detected in every run of sample analyses. The peak area within a chromatographic run was relatively consistent. However, the responses for the very same sample solution varied significantly for every mobile phase preparations, and from one chromatographic system to another. Response of less than 0.05%, by peak area at 230 nm, to well over 0.5% relative to the GSK842879A peak was detected for the same sample solution using different HPLC instrument or a different mobile phase preparation. Further examination of chromatograms revealed that the peak was never present in the blank solutions. In fact, that peak did not appear in injections of any solutions free of GSK842879A. These observations suggested that the peak was likely GSK842879A related.

3.1. Identification of RRT = 1.16 artifact

The ESI accurate mass spectrum of RRT = 1.16 peak displays a singly charged ion at mass to charge ratio (m/z) of 462.0247, as opposed to nominal m/z value of 401 for the protonated molecule of GSK842879A. Fitting of the exact mass (0.43 ppm difference between the measured mass and the calculated mass) and the isotopic pattern (which suggests one copper atom), an elemental composition of $C_{19}H_{19}N_4O_2S_2Cu$ was obtained for the molecular ion at m/z 462.0247. The elemental formula suggests it is probably a $[Cu(II) + GSK842879A–H]^+$ complex ion compared to the active compound GSK842879A. The UV characteristics (top panel in Fig. 1b) of this compound, showing bathochromic shift with significant absorbance beyond wavelength of 350 nm, also suggests RRT = 1.16 peak as the Cu(II)-chelating complex of GSK842879A. The ion at m/z 462 and the protonated molecule of GSK842879A underwent the same fragmentation pathways via the losses of H_2O and H_2S. In addition, these two precursor ions gave rise to the same product ions at m/z 201 and 217. These MS data strongly support that RRT = 1.16 peak is the Cu(II)-chelating complex of GSK842879A. This assumption was further confirmed by the Cu^{2+} doping experiment to be described in Section 3.2.

Multiple structural possibilities of chelating complexation between copper(II) and GSK842879A can be conceived. Two of the most probable structures involve either malonohydrazide in the core (Fig. 2a), or its enolized counterpart, i.e. 3hydrazinyl-3-hydroxyacrylohydrazide (Fig. 2b). Results from the H/D exchange experiment suggested no exchangeable proton in the molecule. Therefore Fig. 2a is a more viable structure for this artifact compound while in the solution phase.

Solids isolated from the RRT = 1.16 artifact peak was brownish. Many distinct infrared bands were noted in the GSK842879A–Cu(II) IR spectrum. The presence of 3456 cm^{-1} band in GSK842879A–Cu(II) complex is a strong indication of existence of –OH functional group. The absorption band centered at 3205 cm^{-1} in GSK842879A molecule, which is attributed to the N–H vibration of the secondary amide, is no longer detected in the GSK842879A–Cu(II) complex. The strong absorption around 1685 cm^{-1} in GSK842879A, due to the carbonyl group, is red-shifted to around 1600 cm^{-1} because of the conjugation with the C=C bond formed from the formation of the Cu(II) complex. The IR data, therefore, suggests Fig. 2b as a more viable structure for the Cu(II) chelate while in the solid phase.

^1H NMR spectrum of GSK 842879A dissolved in $CD_3CN:D_2O$ 60:40 was acquired. Upon addition of 1.5 equivalence of $CuCl_2$ to the solution, the signals were significantly broadened evidencing the formation of the Cu(II) complex. The ^1H NMR spectrum of the isolated RRT = 1.16 compound showed the same broadening signals. Although specific interpretation and assignment to the signals was not possible, the data suggested the isolated compound as a paramagentic complex.

3.2. Confirmation of GSK842879A–Cu(II) chelating complex

Several HPLC experiments were performed to confirm the formation of GSK842879A–Cu(II) chelating complex. Mobile phase A

Fig. 1. Identification and control of the artifact peaks; (a) Artifact peaks at RRT = 1.16 and RRT = 1.49; (b) UV spectra of artifact peaks; (c) HPLC system contaminated with Cu^{2+}, and free of Cu^{2+}; (d) EDTA rinse effect.

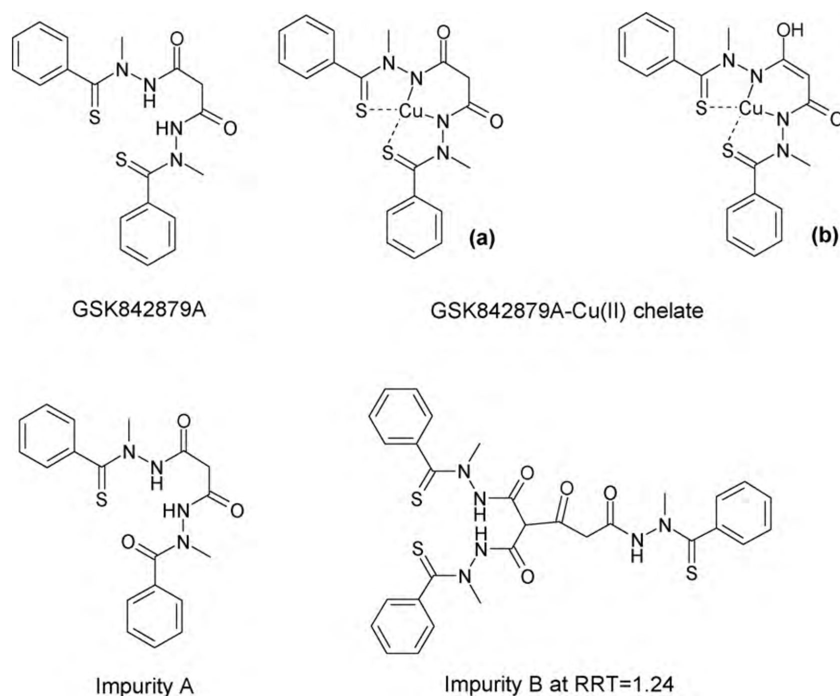

Fig. 2. Structures for GSK842879A, GSK842879A–Cu(II) chelating complex, impurity "A" and RRT = 1.24 impurity.

was spiked with three different concentration levels of Cu^{2+}, beginning at 0.001 mM, followed by 0.005 mM and lastly at 0.01 mM.

HPLC column and system was thoroughly rinsed with 5 mM EDTA in 70:30 water/acetonitrile solution (details of the rinse procedure will be discussed in Section 3.4) to remove carry-over or built-up of Cu^{2+} after each of the spiking experiment. At concentration of 0.001 mM, approximately 0.3% of GSK842879A was converted to its copper chelating complex. When the copper concentration was increased to 0.005 mM, the GSK842879A peak almost completely disappeared, and the chromatogram was predominated by the peak of GSK842879A–Cu(II) chelate.

3.3. Copper chelating complex of impurities

One major drug-related impurity in GSK842879A is impurity A (Figs. 1a and 2). It is a process impurity and also an oxidative degradation product in both drug substance and drug product. The structure of impurity A is very similar to GSK842879A, with modification of thiohydrazide to become hydrazide. In the aforementioned copper spiking study, peak area of impurity A remained unchanged. In another study, sample solutions instead of mobile phase were spiked with Cu^{2+}. Even when the molar ratio of Cu^{2+} to GSK842879A reached 1:10, no change in impurity A response was noted and no extra peak at later eluting time was observed. These results suggested that impurity A, although structurally so similar to GSK842879A, did not form chelating complex with Cu^{2+}.

However, a previously unknown process impurity peak was confirmed to have formed a copper chelating complex (Fig. 1c). It was noted during routine sample analysis that the responses of RRT = 1.49 peak appeared to counter-act with that of RRT = 1.24 peak. Increase in the response of RRT = 1.49 had resulted in decrease of RRT = 1.24 peak, and vise versa. LC/MS study indicated that RRT = 1.24 peak was a process impurity, structurally approximate to that of a 1.5-mer of GSK842879A (impurity B in Fig. 2). In the presence of Cu^{2+}, this impurity was completely sequestered and eluted at RRT = 1.49 as its Cu(II) chelate.

3.4. Analytical control to prevent the formation of Cu(II) chelating artifact peaks

To prevent the formation of the chelating complexes, the sample solution to be analyzed would have to be shielded from any possible Cu^{2+} contamination sources. EDTA is a well-known ligand with high affinity constant forming chelating complexes with many metal ions on 1:1 ratio regardless the charge of the cation ions. The complex of EDTA and Cu^{2+} is stable with formation constant reported as 18.80 [8]. Conceptually, there are three different approaches that strong copper(II) chelating capability of EDTA can be used to control the formation of the encountered artifact peaks. One is to add EDTA to the mobile phase as a scavenger agent so it would trap heavy metals along the chromatographic separation. The second choice is to prepare GSK842879A sample into a suitable solvent that contains appropriate level of EDTA. The last alternative would be using EDTA as a cleansing agent to purge the HPLC system before analysis.

The main goal for us was to achieve control so the artifacts would not form. When 1 mM of EDTA was prepped into mobile phase A, retention time of the drug compound and all of the related impurities remained relatively unchanged. But RRT = 1.16 artifact persistently remained at >0.05% which was above the reporting limit of the drug substance. A second issue with EDTA in mobile phase is lower detection sensitivity because EDTA absorbs light up to 300 nm. Due to these limits, no extensive studies were conducted to control chelating by adding EDTA into mobile phase. The effectiveness of preventing the chelating complex formation by adding EDTA directly into sample solution presumptively depends on relative copper binding affinity of EDTA versus GSK842879A. In the experiment comparing copper binding affinity with EDTA and GSK842879A, EDTA to Cu^{2+} ratio was maintained at 10 to 1, while solutions were prepared at two concentration levels −0.3 mM EDTA to 0.03 mM of Cu^{2+}, and 3 mM of EDTA to 0.3 mM of Cu^{2+}. In both solutions the concentration of GSK842879A was 5 mM. When these solutions were chromatographed on an EDTA rinsed HPLC systems (detailed description in the paragraph following), both artifact peaks were detected. The peak area response for RRT = 1.49 peak was 0.5% from both solutions. However, the response for RRT = 1.16 was increased from 0.63% in solution containing 0.3 mM EDTA/0.03 mM Cu^{2+}, to 5.2% in 3 mM EDTA/0.3 mM Cu^{2+}. These results indicated that vast majority of Cu^{2+} had chelated with GSK842879A, therefore direct addition of EDTA to the sample solution could not prevent GSK842879A from forming its copper chelating complex.

The third approach turned out to be successful, and offered the most desirable outcomes from the project perspectives. The successful control we identified and implemented was to rinse HPLC system utilizing 5 mM of EDTA in a 70:30 water/acetonitrile solution. The entire HPLC system, including the analytical column and solvent delivery lines (with a 50:50 split) was conditioned with the EDTA solution at 2 mL/min flow rate for 60 min. The effect of EDTA rinse is displayed in Fig. 1(d). After the rinse, both artifact peaks were completely removed. Concomitantly, the process impurity at RRT = 1.24 was liberated from Cu(II) complexation and became detected. Our data also suggested that the rinse introduced no detrimental impact on the response of the active and any other impurity peaks, exemplified by preserved baseline features and excellent reproducibility of impurities including impurity A and RRT = 1.24 peak. The lasting effect of EDTA rinse was tested by continuous injections of GSK847879A sample solutions. It was demonstrated that the rinse could sustain continuous analysis for at least up to 100 injections. The method was therefore modified to include this rinse procedure as a step to condition and equilibrate HPLC system.

3.5. Study of potential chelating of GSK842879A with other transition metal ions

Many transition metals are known to form stable cheating complexes with various chelating ligands, the possibility of formation of chelating complexes between GSK842879A and Fe^{3+}, Zn^{2+}, Co^{2+} or Ni^{2+} was examined. The spiked GSK842879A solutions were analyzed to look specifically for late eluting metal-chelating complexes. No extra peaks were detected for the solutions spiked with either Fe^{3+}, or Zn^{2+}, suggesting that GSK842879A does not form chelating complexes with Fe^{3+} or Zn^{2+}, or such complexes were not stable to survive the chromatographic separation. In contrary, GSK842879A was shown to be capable of forming chelates with Co^{2+} and Ni^{2+} as well. These chelates elute at relative retention time of 1.19 and 1.31. After the detection of GSK842879A–Ni(II) or GSK842879A–Co(II) complexes, the EDTA rinse procedure was applied and proved to be effective in removing these two transition metal ions. This is not a surprise since the formation constant of EDTA–Co(II) and EDTA–Ni(II) is 16.31, and 18.62, respectively, comparable to that of EDTA–Cu(II) of 18.80 [8]. Since Co^{2+} and Ni^{2+} are less likely contaminants in the HPLC system, such complexes were never detected in the analysis of GSK842879A samples.

3.6. Investigation of the sources of Cu^{2+} contamination

As stated earlier, conducting analysis of GSK842879A on a HPLC system without applying EDTA rinse always led to the observation of artifact peaks at RRT = 1.16 and RRT = 1.49. Both artifacts stayed fairly constant for injections during a given HPLC run. This implies

that there exists a continuous feed of Cu^{2+}. The copper content in GSK842879A sample and in Milli-Q water was analyzed using ICP-AA, with 0.02 ppm detection limit. Testing results showed no detectable amount of copper.

The residual effect of Cu^{2+} contamination on the column and on the HPLC system was separately examined. The Cu^{2+} contaminated column (in contact with mobile phase A containing 0.03 ppm Cu^{2+} at flow rate of 2 mL/min for over 10 h) was connected to a HPLC system already rinsed with EDTA. HPLC grade water from EMD was used to prepare mobile phase A. An extremely small baseline raise was detected at the retention time of GSK842879A–chelate when sample solution was injected. But the size of the peak was negligible for any meaningful measurement. This indicated that a clean HPLC system combined with Cu^{2+}-free water does not lead to the formation of the chelating artifacts even after the column was deliberately contaminated. On the other hand, a Cu^{2+} contaminated HPLC instrument (in contact with mobile phase A containing 0.03 ppm Cu^{2+} at flow rate of 2 mL/min for over 10 h) was used in combination of an EDTA rinsed column. Again HPLC grade water from EMD was used to prepare mobile phase A. At this combination, the GSK842879A–Cu(II) chelating peak was readily detected. Although, the peak became smaller as more injections were made, it remained almost constant after the first few injections. This allowed us to conclude that residual effect might be lasting after HPLC system became contaminated. It also explained why extensive washing of HPLC system with mobile phase or other solvents was not particularly helpful in eliminating the chelate artifacts, especially GSK842879A–Cu(II) chelate peak.

The peak area of chelates was affected by amount of Cu^{2+} in the mobile phase and Cu^{2+} residue in HPLC system. Cu^{2+} in water from different sources and Cu^{2+} residue in different HPLC instrument could vary. As a result, a change in mobile phase preparation or switch of HPLC instrument could lead to the formation of both artifact peaks in variable sizes.

4. Conclusion

Common artifact peaks in HPLC separation can be relatively easily controlled by carefully maintaining LC instrument and column, and by controlling quality of mobile phase components and avoiding contamination of sample solutions. Unusual artifact peaks have to be studied, their formation understood, and control methodology developed in order to improve the reliability and robustness of the HPLC methods. Our investigation of the chromatographic artifact peaks in the HPLC analysis of GSK842879A suggested built-up of Cu^{2+} in HPLC system or contamination of Cu^{2+} in mobile phase as the root cause. The RRT = 1.16 artifact was identified as the Cu(II) chelating complex with GSK842879A, and RRT = 1.49 artifact as the Cu(II) chelating complex with a process impurity (impurity B). A rinsing procedure using 5 mM of EDTA was developed and demonstrated to have successfully removed Cu^{2+} contamination and therefore the formation of both chelating artifacts. The rinse was also demonstrated to introduce no detrimental impact on the response accuracy of the active drug compound and all impurity peaks. Implementation of the rinse procedure in the method as HPLC system conditioning and equilibrating step has successfully ensured accurate determination for content of GSK842879A and its related impurities.

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