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Simultaneous quantitation of trace level hydrazine and acetohydrazide in pharmaceuticals by benzaldehyde derivatization with sample 'matrix matching' followed by liquid chromatography–mass spectrometry

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ABSTRACT

Hydrazine and acetohydrazide are potential genotoxins and therefore need to be controlled in APIs and drug products to ppm levels for patient safety in cases where there is a reasonable probability of either of them being present. They are structurally related and could both be formed in the same chemical process under certain circumstances. However, no previous studies have reported simultaneous trace level quantification of these two compounds. Herein, a chemical derivatization scheme using benzaldehyde followed by LC–MS analysis is presented to address that need. During method development, unexpectedly high recoveries were encountered and presented a major challenge. A systematic investigation was undertaken to understand the benzaldehyde derivatization reaction and determine the underlying causes of the unacceptable recovery. It was found that this was due to the presence of the counter ion of the API in the sample matrix. Employing a 'matrix matching' sample preparation strategy, which involved acidifying the derivatization reaction medium with benzoic acid, gave similar reaction rates for the chemical derivatization in the presence and absence of the API salt and accordingly more consistent recoveries. Resultantly, a robust method for simultaneous quantification of hydrazine and acetohydrazide (1–100 ppm) was successfully developed and validated.

1. Introduction

Hydrazine and acetohydrazide are common reagents for the synthesis of various APIs in pharmaceutical R&D [1]. Hence, they can be potential impurities in the final pharmaceutical products. Due to their high reactivity, they may induce mutagenesis upon intake by patients [2,3]. According to the ICH M7 guidance, genotoxic impurities need to be controlled in APIs and/or drug products typically at parts-per-million (ppm) levels [4].

Due to their high reactivity, direct quantification of hydrazine and acetohydrazide is often complicated by the sample matrices including active pharmaceutical ingredient (API) itself and/or impurities therein [5]. Therefore, a chemical derivatization based methodology is more suitable for their quantification. Several studies employing various derivatization reagents and analytical techniques have been reported for quantifying hydrazine at low ppm levels, including HPLC [6–10], GC [11,12], GC–MS [5,13–15], and LC–MS/MS [16,17]. For acetohydrazide, however, there is no trace analysis method in the literature for accurate quantitation in pharmaceuticals at low ppm levels. The objective of this study was to develop a robust method for analyzing both hydrazine and acetohydrazide simultaneously.

Because of the structural similarity, the previously developed chemical derivatization strategies for hydrazine could be adopted for acetohydrazide. Acetone [1,5,11], benzaldehyde [1,18–20] hydroxybenzaldehydes [1,21], 2,3-naphthalene dicarboxaldehyde [22–25] and several others [1,25] have been reported as effective trapping reagents for the highly reactive hydrazine. Acetone derivatization was not selected because the polarity of the derivatization product of acetohydrazide could adversely impact its vaporization under head space GC conditions and impact method sensitivity [1]. Compared with the hydroxybenzaldehydes that contain electron donating group on the benzene rings, benzaldehyde is more reactive, which would improve the derivatization reaction with the less reactive acetohydrazide and was therefore chosen as the derivati-

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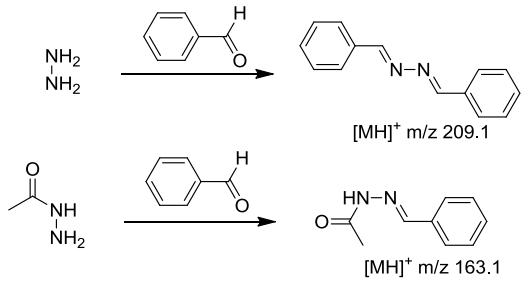

Fig 1. Derivatization reactions of hydrazine and acetohydrazide with benzaldehyde.

zation reagent. As shown in Fig. 1, the derivatization products are 1,2-dibenzylidenehydrazine and benzylideneacetohydrazide for hydrazine and acetohydrazide, respectively. Both of these derivatization products are amenable to reversed-phase HPLC and are easily ionized by electrospray ionization (ESI) in the positive ionization mode. Mass spectrometry is required to achieve the low ppm quantification limits (per the regulatory requirements) and the needed additional specificity when analyzing multiple analytes with similar properties simultaneously [26].

In quantitative analysis of trace level pharmaceutical impurities, calibration standards are usually prepared using pure analytes dissolved in pure solvents given that a blank sample matrix (an impurity-free API or drug product) is typically unavailable. Method accuracy is evaluated by a spiking recovery experiment that compares the analyte peak response of a spiked sample to that of a pure standard at the same concentration. Nevertheless, high concentration of active ingredients in real samples creates the difference in matrices between pure standards and samples for the analytes of interest. This difference can lead to higher or lower responses for the target analyte(s) in samples than that in the pure standard causing unacceptable recoveries [27,28]. This matrix effect issue can be especially problematic when derivatization is involved in the sample preparation because derivatizing reagents can also react with other species in the sample causing a false positive response, or the derivatization reaction might fail to proceed to completion resulting in a false negative response [29,30]. Such a matrix effect issue was encountered in our effort to develop a method for simultaneous quantification of hydrazine and acetohydrazide in an API with targeted limit at 10 ppm (The proposed API's dose is 0.1 g/day and the clinical trial duration is >12 MN. The acceptable daily intake for this study time period (>12 MN) is 10 μg/day based on ICH M7, therefore the limit is expected to be 10 (μg/day)/0.1 (g/day) = 100 ppm. Our method goal, however, was aiming at 10 ppm to cover a potential dose increase). In this case, both of these compounds were implicated as potentially being present in the chemical synthesis of an API: acetohydrazide is a reagent for synthesizing the API and hydrazine is a potential impurity and degradation product of acetohydrazide. To solve the issue, we developed a matrix-matching strategy, which involves chemically modifying the analyte matrix in standard solutions to match that in real samples with intend to minimize or eliminate the matrix difference for the analyte. The subsequent incorporation of the sample matrix-matching enabled a full validation of the derivatization method.

2. Material and methods

2.1. Reagents

Hydrazine monohydrade, (N_2H_4, 64–65%), benzaldehyde (99.5%), benzoic acid (BA, 99.5%), benzenesulfonic acid (BSA, 98%), and HPLC grade acetonitrile (MeCN), methanol and formic acid (FA) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetohydrazide (97%) was obtained from Combi-Blocks, Inc (San Diego, CA, USA). Water was from a Millipore Milli-Q water-purification system (Bedford, MA, USA). All APIs, GSK21XXXX (API_1), GSK52XXXX (API_2), SB68XXXX (API_3) and GSK219XXXX (API_4), were prepared in house at GlaxoSmithKline (King of Prussia, PA, USA).

2.2. LC–MS

An Agilent LC–MS system consisting of a 1290 LC coupled with a 6150 MSD (Santa Clara, CA, USA) with ESI was operated in the positive ionization mode with the capillary voltage set to 3 kV. The fragmentor (cone voltage) was set to 70 V. The drying gas flow was set to 12 L/min with a temperature of 350 °C. The nebulizer pressure was set to 40 psi. For the single-ion-monitoring (SIM), ions at *m/z* 209.2 and *m/z* 163.2 were monitored for 1,2-dibenzylidenehydrazine and benzylideneacetohydrazide (derivatization products of hydrazine and acetohydrazide), respectively. A Phenomenex Luna C18 (2) column (100 Å, 3 μm, 150 × 4.6 mm) was used. The mobile phases A and B were 0.1% formic acid in H_2O and 0.1% formic acid in MeCN, respectively. The chromatographic separation was achieved using the following gradient elution: 0–3 min, 35% B; 3–7 min, ramping from 35% to 90% B; 7–10 min, 90% B, with a constant flow rate of 1.0 mL/min. The column temperature was kept at 40 °C. The typical injection volume was 5 μL.

2.3. Preparation of solutions

2.3.1. Sample diluent

MeCN-H_2O (70:30, v/v) was used as the blank solution and sample diluent.

2.3.2. Derivatization reagent solutions

The base derivatization reagent solution **A** of 0.4% (v/v) benzaldehyde (excessive in molarities) in sample diluent was prepared typically by transferring 4 mL of benzaldehyde into a 1000 mL volumetric flask and making up to the volume using the above sample diluent. Other derivatization solutions with different acid modifiers at various concentrations were then prepared using solution **A**. For example, the 50 mM benzoic acid fortified derivatization solution was prepared by dissolving 610.6 mg of benzoic acid in 100 mL of solution **A**.

2.3.3. Hydrazine and acetohydrazide standard solutions

The 1 mg/mL stock standard mix of hydrazine and acetohydrazide was prepared by adding 0.154 mL of hydrazine and 10.0 mg of acetohydrazide into a 10-mL volumetric flask and making up to the volume using the sample diluent. The 4 μg/mL standard mix of hydrazine and acetohydrazide was prepared by transferring 0.100 mL of the 1 mg/mL stock into a 25-mL volumetric flask and diluting to volume with diluent. Other standard solutions were prepared in a similar manner at concentrations of 400, 80, 40, 20, 4, 0.8, and 0.4 ng/mL. The standard linearity was evaluated from 0.4 to 80 ng/mL (0.1–20 ppm) for hydrazine and 4–400 ng/mL (1–100 ppm) for both hydrazine and acetohydrazide, relative to a 4 mg/mL sample.

2.4. Chemical derivatization procedures

2.4.1. Derivatization of the standards

To perform the derivatization reaction of the standard at 40 ng/mL, 10 μL of the 4 μg/mL standard mix of hydrazine and acetohydrazide was transferred into each 2-mL HPLC vial containing 1 mL of the desired derivatization reagent solution (with acid modifier prepared from solution A as indicated above). The vials were