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Hydroxylamine Analysis by HPLC Using Pre-Column Derivatization: A Literature Review on Pharmaceutical Applications

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Abstract: *Hydroxylamine (NH_2OH) and its salts are commonly used as reducing agents and intermediates in the synthesis of active pharmaceutical ingredients (APIs), but due to their potential genotoxicity, they must be controlled at trace (low ppm) levels. Direct analysis of hydroxylamine is challenging because it is small, highly polar, lacks a chromophore, and contains no carbon atoms, making conventional HPLC–UV or GC methods unsuitable without derivatization.*

This review summarizes reported high-performance liquid chromatographic (HPLC) methods based on pre-column derivatization, in which hydroxylamine is converted into stable UV-absorbing derivatives, such as benzaldoxime (via benzaldehyde) or FMOC derivatives (via 9-fluorenylmethyl chloroformate, FMOC-Cl). Key aspects discussed include derivatization chemistry, chromatographic conditions, sample preparation strategies, and validation parameters such as linearity, sensitivity, precision, and robustness. Applications in APIs including Vorinostat, Zileuton, Relugolix, and Febuxostat are highlighted.

The advantages and limitations of existing methods are critically evaluated in the context of regulatory requirements for genotoxic impurity control. Future perspectives focus on developing more generic, automated, and LC–MS compatible methods to improve sensitivity, selectivity, and compliance in pharmaceutical quality control..

Keywords: Hydroxylamine; genotoxic impurity; pre-column derivatization; HPLC-UV; benzaldoxime; FMOC-Cl; pharmaceutical analysis

I. INTRODUCTION

Hydroxylamine and its inorganic salts, particularly hydroxylamine hydrochloride, are frequently used as key reagents in synthetic routes for pharmaceutical intermediates and final APIs, which creates a realistic risk that residual hydroxylamine may remain in the drug substance if the process is not adequately controlled (Kumar et al., 2019) [2]. Because hydroxylamine is classified as a mutagenic or potentially genotoxic impurity, regulatory guidance requires that its level in drug substances be suitably controlled, often at or below permitted daily exposure values based on the threshold of toxicological concern (Kumar et al., 2019; Role of analytical methods)[2],[3]. However, the physicochemical properties of hydroxylamine—high polarity, low molecular weight, absence of a chromophore, and inorganic character—render direct UV or standard chromatographic detection difficult, especially in the presence of complex pharmaceutical matrices that can produce substantial background interference (Kumar et al., 2019)[2]. Consequently, pre-column derivatization followed by reversed-phase HPLC has emerged as a practical and sensitive strategy for routine quantification of hydroxylamine in APIs.

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II. ANALYTICAL CHALLENGES IN HYDROXYLAMINE DETERMINATION

Several inherent properties of hydroxylamine make its determination in pharmaceutical matrices technically demanding. First, hydroxylamine is a small, highly polar molecule that contains no carbon atoms and no significant chromophore, which means it produces negligible signal in conventional HPLC–UV systems unless converted into a UV-active derivative (Kumar et al., 2019) [2]. Second, hydroxylamine is a strong nucleophile and reducing agent that can undergo side reactions with carbonyl-containing excipients or APIs, particularly under alkaline conditions that are commonly used for derivatization reactions, leading to potential analyte loss or matrix-derived interferences (Song et al., 2016) [4]. Third, drug substances and their related impurities often exhibit strong UV absorbance and complex chromatographic behaviour, which can overshadow any direct response from hydroxylamine if co-elution occurs (Kumar et al., 2019) [2]. These challenges collectively motivate the use of selective derivatization strategies and appropriate sample pretreatment to isolate hydroxylamine from interfering components prior to HPLC analysis (Song et al., 2016) [4].

III. CONCEPT AND RATIONALE OF PRE-COLUMN DERIVATIZATION

Pre-column derivatization involves reacting hydroxylamine with a suitable reagent before chromatographic injection to form a derivative that is more hydrophobic and possesses a strong UV-absorbing or fluorescent chromophore (Song et al., 2016) [4]. In the context of pharmaceutical analysis, this approach serves multiple purposes: it enhances detectability, enables retention on reversed-phase columns, and can improve separation from inorganic and low-molecular-weight components of the matrix (Kumar et al., 2019) [2]. For hydroxylamine, reported HPLC methods have mainly focused on two derivatization chemistries: (i) formation of FMOC-hydroxylamine via reaction with 9-fluorenylmethyl chloroformate (FMOC-Cl) and (ii) formation of benzaldoxime via reaction with benzaldehyde or related aromatic aldehydes (Song et al., 2016; Venkata Ramana et al., 2018; Zang et al., 2024)[4],[5],[6].

An effective derivatization reagent must react rapidly and selectively with hydroxylamine under conditions compatible with the API, forming a stable product that can withstand routine sample handling and chromatographic analysis (Song et al., 2016; Zang et al., 2024)[4],[6]. In addition, excess reagent and reagent-derived by-products must not interfere with detection of the analyte derivative, and the derivatization conditions should avoid degradation of sensitive drug substances (Song et al., 2016)[4].

IV. FMOC-CL DERIVATIZATION METHODS IN APIS

Song and co-workers described a selective and sensitive HPLC method for trace analysis of hydroxylamine in the APIs Vorinostat and Zileuton using pre-column derivatization with FMOC-Cl (Song et al., 2016)[4]. Their study highlighted a key problem: both hydroxylamine and the APIs can react with FMOC-Cl, creating competition and potential degradation of the drug substances under strongly alkaline derivatization conditions (Song et al., 2016) [4]. To address this, the authors implemented an innovative sample pretreatment in which the API was first precipitated, allowing hydroxylamine to remain in the supernatant where it could be selectively derivatized, thereby minimizing API degradation and competitive reactions (Song et al., 2016)[4].

The derivatization conditions were extensively optimized under both hydrous and anhydrous environments, varying parameters such as reaction time and temperature, concentrations and volumes of FMOC-Cl and sodium borate, base type, and pH, to maximize the response of FMOC-hydroxylamine relative to FMOC-API adducts (Song et al., 2016)[4]. LC–TOF/MS and LC–MS/MS were employed to confirm the structures of the various FMOC derivatives formed, enabling a mechanistic understanding of the derivatization process that guided optimization (Song et al., 2016)[4]. The final method afforded limits of detection and quantitation around 0.01 and 0.03 ppm, respectively, with satisfactory precision and accuracy, demonstrating that classical FMOC derivatization coupled to HPLC–UV can meet regulatory sensitivity requirements for genotoxic impurity control (Kumar et al., 2019; Song et al., 2016)[2],[4].

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V. BENZALDEHYDE–BENZALDOXIMEDERIVATIZATION FOR RELUGOLIX AND OTHER APIS

A second major derivatization approach is based on the reaction of hydroxylamine with benzaldehyde to form benzaldoxime, which incorporates an aromatic ring with strong UV absorbance and adequate hydrophobicity for reversed-phase separation (Venkata Ramana et al., 2018; Zang et al., 2024). Zanget al.[5],[6] developed a pre-column derivatization HPLC–UV method using benzaldehyde as the derivatizing reagent for determination of hydroxylamine hydrochloride in Relugolix drug substance (Zang et al., 2024) [6]. In their procedure, hydroxylamine hydrochloride reacts with benzaldehyde in the presence of triethylamine to generate benzaldoxime; kinetic evaluation showed that the reaction reached completion within 5 minutes, and the derivative peak remained stable for at least 120 minutes, with 30 minutes chosen as a practical derivatization time (Zang et al., 2024)[6].

Chromatographic separation in the Relugolix method was performed on a C18 column (YMC-Pack ODS-A, 150 × 4.6 mm, 5 µm) using an isocratic mobile phase of acetonitrile and 0.01 mol/L potassium dihydrogen phosphate buffer adjusted to pH 2.3 in a 35:65 (v/v) ratio, at a flow rate of 1.0 mL/min, column temperature of 40 °C, injection volume of 10 µL, and UV detection at 254 nm (Zang et al., 2024)[6]. Under these conditions, the benzaldoxime peak was well separated from blank and matrix peaks, and specificity studies showed no interference from either derivatization reagents or Relugolix itself (Zang et al., 2024)[6]. Similar benzaldehyde-based derivatization principles have also been applied to other APIs such as Febuxostat, where a sensitive and validated HPLC method was reported for hydroxylamine determination at low µg/g levels (Venkata Ramana et al., 2018)[5].

VI. GENERIC HPLC DERIVATIZATION METHODS FOR HYDROXYLAMINE

Beyond API-specific applications, Kumar et al. reported a generic HPLC–UV derivatization method for determination of hydroxylamine in drug substances, emphasizing its applicability across different molecules (Kumar et al., 2019)[2]. Their method also relied on pre-column derivatization followed by reversed-phase HPLC–UV, achieving a detection limit of 0.01 ppm and a quantitation limit of 0.03 ppm, with signal-to-noise ratios greater than 3 and 10, respectively (Kumar et al., 2019)[2]. Validation in accordance with ICH Q2(R1) demonstrated good linearity, precision, accuracy, and robustness, supporting its use as a generic screening method for hydroxylamine during pharmaceutical process development and routine drug substance release (Kumar et al., 2019)[2].

Venkata Ramana and colleagues developed and validated a benzaldehyde-based derivatization HPLC method for hydroxylamine as a mutagenic impurity in Febuxostat drug substance, confirming that this strategy can be adapted to other APIs with appropriate optimization of chromatographic conditions and sample preparation (Venkata Ramana et al., 2018)[5]. Collectively, these studies show that derivatization-HPLC approaches are flexible and can be tailored to different chemical and matrix environments while maintaining the sensitivity necessary for genotoxic impurity control.

VII. SAMPLE PREPARATION AND MATRIX MANAGEMENT

Sample preparation is a critical element for accurate hydroxylamine quantification because of potential API reactivity and matrix interferences. In the FMOC-Cl method for Vorinostat and Zileuton, Song et al. used an API precipitation step prior to derivatization, which effectively removed much of the drug substance and related reactive species from the solution, thereby isolating hydroxylamine in the supernatant for selective derivatization (Song et al., 2016) [4]. This strategy mitigated both degradation of sensitive APIs and the formation of competing FMOC derivatives, significantly improving method selectivity and robustness (Song et al., 2016)[4].

In the benzaldoxime method for Relugolix, Zang et al. prepared sample solutions by dissolving defined amounts of API, adding benzaldehyde solution and triethylamine, and diluting to volume with water before allowing the derivatization to proceed at room temperature (Zang et al., 2024)[6]. Separate blank, standard, sample, and spiked sample solutions were analyzed to confirm specificity and absence of interfering peaks at the benzaldoxime retention time (Zang et al., 2024)[6]. Similar approaches—careful control of sample dissolution, reagent volumes, and reaction times—are evident in benzaldehyde-based methods for Febuxostat and other drug substances, underscoring the importance of matrix-optimized sample preparation (Venkata Ramana et al., 2018)[5].

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VIII. TYPICAL HPLC CONDITIONS FOR HYDROXYLAMINE DERIVATIVES

Despite differences in specific applications, reported HPLC methods for hydroxylamine derivatives share several common chromatographic features. Most use C18 reversed-phase columns (e.g., 150 × 4.6 mm, 5 µm) and binary mobile phases composed of acetonitrile and aqueous phosphate buffers, with the aqueous phase often adjusted to acidic pH (around 2–3) to improve peak shape and reproducibility for aromatic derivatives (Song et al., 2016; Venkata Ramana et al., 2018; Zang et al., 2024) [4],[5],[6]. Detection wavelengths are selected according to the chromophore introduced by derivatization: approximately 254 nm for benzaldoxime derivatives and slightly longer wavelengths for FMOC derivatives, reflecting their specific absorption maxima (Song et al., 2016; Zang et al., 2024)[4],[6].

The Relugolixbenzaldoxime method employed isocratic elution with acetonitrile:phosphate buffer (35:65, v/v) at a flow rate of 1.0 mL/min, column temperature of 40 °C, and 10 µL injection volume, with a total run time of 30 minutes (Zang et al., 2024)[6]. FMOC-based methods often use gradient elution to resolve multiple FMOC-tagged species, although specific gradient programs differ among laboratories and are tailored to each matrix (Song et al., 2016) [4]. Across methods, baseline separation of the hydroxylamine derivative from excess reagent, reagent degradation products, and matrix peaks is emphasized as essential for method specificity and reliability.

IX. VALIDATION CHARACTERISTICS AND ANALYTICAL PERFORMANCE

Derivatization-HPLC methods for hydroxylamine are generally validated in accordance with ICH Q2(R1), with parameters including specificity, linearity, accuracy, precision, LOD, LOQ, robustness, and solution stability (Kumar et al., 2019; Song et al., 2016; Venkata Ramana et al., 2018; Zang et al., 2024)[2],[4],[5],[6].Zang et al. reported a limit of quantitation of 12 ppm for hydroxylamine hydrochloride in Relugolix API, with a linear range of 12–360 ppm and correlation coefficient greater than 0.99; the RSD for LOQ peak areas in six replicate injections was 1.11%, and accuracy studies at 50%, 100%, and 150% spike levels yielded recoveries in the range of approximately 94–97% with an overall RSD of 1.93% (Zang et al., 2024)[6].

Kumar et al. demonstrated that their generic HPLC–UV derivatization method could detect hydroxylamine down to 0.01 ppm and quantify it down to 0.03 ppm, with adequate signal-to-noise ratios and acceptable precision for both repeatability and intermediate precision studies (Kumar et al., 2019). VenkataRamana et al[2],[5]. reported LOD and LOQ values of approximately 1.7 and 5.0 µg/g, respectively, for benzaldehyde-derivatized hydroxylamine in Febuxostat drug substance, and described the method as specific, sensitive, accurate, and precise for routine testing (Venkata Ramana et al., 2018)[5]. Collectively, these validation data indicate that pre-column derivatization HPLC methods are capable of meeting stringent sensitivity and reliability requirements associated with genotoxic impurity control.

X. COMPARISON WITH ALTERNATIVE ANALYTICAL APPROACHES

Although derivatization-based HPLC–UV is prominent in the pharmaceutical literature, other analytical techniques have been explored for hydroxylamine determination. Electrochemical detection, ion chromatography, and GC-based methods—often with derivatization to form volatile oxime derivatives—have been applied mainly in environmental or process monitoring contexts (Kumar et al., 2019; Venkata Ramana et al., 2018)[2],[5]. For instance, GC–headspace–MS methods using acetone derivatization have achieved very low detection limits for hydroxylamine in penicillamine drug substance, but require specialized instrumentation and more complex method set-up compared with conventional HPLC–UV (Kumar et al., 2017)[1].

Liquid chromatography–mass spectrometry (LC–MS and LC–MS/MS) methods without derivatization have also been described for hydroxylamine, offering higher selectivity and potential for multiplexed genotoxic impurity panels (Kumar et al., 2017)[1]. However, many QC laboratories remain HPLC–UV-centric due to cost and infrastructure constraints; thus, pre-column derivatization coupled with HPLC–UV provides a pragmatic compromise between sensitivity, selectivity, and operational feasibility (Kumar et al., 2019; Role of analytical methods) [2],[3]. Future trends

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are likely to include broader adoption of LC–MS/MS, green derivatization reagents, and automated sample preparation workflows.rroij,[4]

XI. REGULATORY CONTEXT AND QUALITY CONTROL IMPLICATIONS

Regulatory expectations for mutagenic and genotoxic impurities, as described in ICH M7 and related guidance, require drug manufacturers to assess and control impurities such as hydroxylamine at levels that do not pose unacceptable cancer risk (Role of analytical methods)[3]. A review on analytical methods for detection of genotoxic impurities emphasizes that HPLC, often in combination with derivatization, is a central technique because many genotoxic species lack strong chromophores or straightforward ionization behaviour (Role of analytical methods) [3]. The derivatization-HPLC methods discussed in this review provide validated, stability-indicating procedures capable of routine implementation in QC laboratories for batch release and stability testing of APIs containing hydroxylamine in their synthetic routes (Kumar et al., 2019; Song et al., 2016; Venkata Ramana et al., 2018; Zang et al., 2024). Rroij,[2][4],[5],[6].

Given the increasing regulatory scrutiny on genotoxic impurities, robust, sensitive, and well-characterized methods for hydroxylamine quantification are integral to demonstrating process understanding, impurity control, and product safety throughout the product lifecycle (Kumar et al., 2019; Role of analytical methods) [2],[3].

XII. CONCLUSION

This review critically examines the analytical approaches reported for the determination of hydroxylamine as a potential genotoxic impurity in pharmaceutical substances. Existing HPLC and LC–MS/MS methods demonstrate adequate sensitivity at low ppm levels; however, most are highly API-specific and require individual optimization for different matrices.

Future research should focus on developing more generic platform methods using broadly applicable derivatization chemistries or direct LC–MS/MS strategies that can be transferred across APIs with minimal re-optimization. Integration of Analytical Quality by Design (AQbD) principles may further enhance method robustness and lifecycle management. Additionally, greener derivatization reagents, automated on-line systems and multi-analyte genotoxic impurity panels combined with high-efficiency UHPLC or LC–MS/MS platforms could significantly improve analytical efficiency and regulatory compliance in modern pharmaceutical development.

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