Fast and Simple Determination of 3-Aminopiperidine without Derivatization Using High Performance Liquid Chromatography-Charged Aerosol Detector with an Ion-Exchange/Reversed-Phase Mixed-mode Column

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A sensitive non-derivatization method for the determination of the highly polar compound 3-aminopiperidine was developed using a mixed-mode column combined with a charged aerosol detector (CAD). Chromatographic conditions, including the type of detector, separation mode, and mobile phase composition, were optimized to achieve high sensitivity towards and sufficient retention of 3-aminopiperidine. Compared to the precolumn derivatization UV method, the current method showed higher recovery and greater simplicity. High sensitivity (LOQ <2.73 μg mL⁻¹) and good precision (RSD of peak area <2%) were also observed in the current method. Furthermore, the parameters such as buffer solution and column bleed that affected the sensitivity of the CAD were investigated. Finally, the current method was applied for the determination of 3-aminopiperidine in linagliptin samples. This is a new non-derivative for the determination of 3-aminopiperidine, and constitutes a novel application of the CAD for the quantitative analysis of highly polar basic compounds.

Keywords 3-Aminopiperidine, non-derivatization, charged aerosol detector, linagliptin

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compounds may be a limitation in high concentrations of the organic solvent, which is usually used as the initial mobile phase in HILIC. Mixed-mode column is another choice for the separation mode, type of columns, and mobile phase (CAD) was developed for the analysis of 3-aminopiperidine.23–26 Such columns are usually used with volatile buffer solutions; thus, they can be used with universal detectors for the analysis of polar basic compounds that lack UV-absorbing chromophores.

3-Aminopiperidine is a highly polar basic compound and proves difficult for detection by a UV detector. It is an important starting material in the synthesis of linagliptin (Fig. 1), which has been approved as a DPP-4 inhibitor for the treatment of type II diabetes.27 The determination of 3-aminopiperidine in the drug sample is of critical importance for quality control. Few methods have been reported for the analysis of such a sample. Babu et al.11 used a chiral HPLC-UV method, involving a precolumn derivatization technique with para-toluene sulfonyl chloride (PTSC), to estimate the enantiomeric impurity of 3-aminopiperidine. However, the sample required derivatization before analysis. Another method was proposed by Meek et al., using HPLC coupled with a refractive index detector to directly assay the 3-aminopiperidine dihydrochloride and its enantiopurity.28 In the current study, a method involving a mixed-mode column combined with a charged aerosol detector (CAD) was developed for the analysis of 3-aminopiperidine in linagliptin. Chromatographic conditions, including the separation mode, type of columns, and mobile phase composition, were optimized. Moreover, different types of detectors, CAD and UV detector, were compared. Finally, the optimized method was used for the determination of 3-aminopiperidine in linagliptin.

**Experimental**

**Apparatus and reagents**

The chromatographic system contained an LPG-3400SD pump, WPS-3000 TSL autosampler, TCC-3000RS column oven, and Veo RS CAD. Data were collected and analyzed by Chromeleon Ver. 7.2. All the mentioned instruments and the workstation were from Thermo Fisher (Sunnyvale, CA, USA). The Acclaim Trinity P1 column (150 × 3.0 mm, 3 μm), Acclaim C18 column (150 × 4.6 mm, 5 μm), and Acclaim XAmide (150 × 4.6 mm, 5 μm) were also from Thermo Fisher (Sunnyvale, CA, USA). The Chiralpak AD-H (250 × 4.6 mm, 5 μm) column was from Daicel (Tokyo, Japan).

HPLC-grade acetonitrile, methanol, and ethanol were purchased from TEDIA (Ohio, USA). Ammonium formate (99.999%) was obtained from Acros (NJ, USA). Ammonium acetate (98%) and diethylamine (analytical grade) were purchased from Sigma (St. Louis, MO, USA). Ultra-pure water was produced in-house (Millipore, Advantage A10, USA). Linagliptin was provided by JiangSu Deyuan Pharmaceutical Co., Ltd. (JiangSu, China). 3-Aminopiperidine dihydrochloride was purchased from J&K Scientific (98%, China). Nitrogen (99.999%) was purchased from Shengtang (Tianjin, China). All the other chemicals and reagents used were of analytical grade, unless indicated otherwise.

**Preparation of standard stock solution and working solutions**

The standard stock solution of 3-aminopiperidine was prepared by weighing 17.29 mg of the 3-aminopiperidine dihydrochloride in a 10 mL volumetric flask. Then 1 mL was transferred to an individual 20 mL volumetric flask and diluted to volume with water, giving a concentration of 50 μg mL⁻¹ of 3-aminopiperidine. The standard stock solution was stored at room temperature and the required concentrations of working solutions were then prepared by serial dilution using standard stock solution with water.

**Preparation of sample solution**

An appropriate amount of linagliptin was transferred separately into individual 10 mL volumetric flasks, diluted to volume with methanol, giving a final concentration of 5 mg mL⁻¹. The sample solution was stored at room temperature.

**Chromatographic conditions**

Chromatography was performed using an Acclaim Trinity P1 column. Solvent A was acetonitrile, solvent B was 30 mM ammonium formate, and solvent C was water. For the analysis of linagliptin, mobile phase A (ACN) increased from 10 to 50% while mobile phase C (water) decreased from 60 to 20% and mobile phase B held constant at 30% for 16 min. The flow rate was 0.7 mL min⁻¹. The injection volume was 20 μL and the column oven temperature was 30°C. The CAD was set at an evaporation temperature of 55°C and a gas pressure of 62 psi. The acquisition frequency was 10 Hz. The mobile phases used for Acclaim C18 and Acclaim XAmide were methanol:water (5:95, v/v) and acetonitrile:water (95:5, v/v), respectively, and the other conditions were the same as for the Acclaim Trinity P1 column.

**Chromatographic conditions for precolumn derivatization method**

The precolumn derivatization analysis was performed on a Chiralpak AD-H. The mobile phase consisted of 0.1% diethyl amine in ethanol. The detection wavelength was set at 228 nm. The column temperature was maintained at 27°C. The injection volume was 5 μL. The flow rate was 0.5 mL min⁻¹.

**Results and Discussion**

**Effects of detector and column type**

UV detectors are the most widely used detectors in the pharmaceutical industry, because they present a wide range of linearity, good repeatability, and high sensitivity.3,11,29 For the detection of 3-aminopiperidine, however, UV detectors provide a poor response, because 3-aminopiperidine has no chromophores (see Fig. S1, Supporting Information). The CAD is reported to be a universal detector and provides a good response towards non-volatile compounds.30,32 Therefore, the CAD was used for optimizing the chromatographic conditions for 3-aminopiperidine in the experiments described in the following sections.

3-Aminopiperidine was first analyzed on a conventional ODS column, Acclaim C18, using methanol:water (5:95, v/v) as the mobile phase (Fig. 2A). The retention for the compound in this case was insufficient for good separation, because of its high

![Chemical structures of 3-aminopiperidine and linagliptin.](image-url)
hydrophilicity. Trifluoroacetic acid (TFA) or formic acid (FA) is widely used as a volatile additive and can have ion-pairing capability in reversed-phase chromatographic conditions. Then, mobile phases containing 0.05% TFA and 0.1% FA were used on Acclaim C18 columns, respectively, but there was still no sufficient retention for 3-aminopiperidine to be well separated (see Fig. S2, Supporting Information). Ion-pairing reagents such as hexane sulfonate can enhance the retention of polar basic compounds.\textsuperscript{33} However, such reagents usually modify the columns and lead to poor repeatability of the columns. Furthermore, they are incompatible with universal detectors such as the CAD. Hence, an ion-pairing reagent was not used in this study. HILIC is an alternative to RPLC for the separation of polar compounds.\textsuperscript{34} We tested the separation of 3-aminopiperidine on an HILIC column, Acclaim XAmide. A much longer retention was observed on this column (Fig. 2B) than on the C18 column. Apart from HILIC columns, mixed-mode columns such as Trinity P1 have been reported for the analysis of polar compounds.\textsuperscript{23} As shown in Fig. 2C, sufficient retention of the target compounds can also be obtained on Trinity P1.

Although both HILIC and mixed-mode analyses can be used to achieve sufficient retention for 3-aminopiperidine, the peak areas and signal-to-noise ratios obtained with these two methods are very different. In the HILIC mode, a high percentage of organic solvent is used, to afford better transport efficiency of the nebulizer. Consequently, a greater number of particles reach the chamber and a larger peak area is obtained, as compared to the case when the mobile phase has low concentrations of organic solvents.\textsuperscript{30,35,36} However, bleeding of the HILIC column is much higher than that for the other columns (Fig. 3A), and the CAD is sensitive to column bleed.\textsuperscript{37} In other words, high column bleed will lead to high background, large baseline noise, and a low signal-to-noise ratio. Compared to the case of the HILIC column, a mixed-mode column shows much lower bleed (Fig. 3B). A lower concentration of organic solvent was used in the mixed-mode column than in the HILIC mode, leading to low response for 3-aminopiperidine; however, a higher signal-to-noise ratio was obtained in the mixed-mode ($S/N = 154$) method than that in the HILIC mode ($S/N = 130$), because of a lower baseline noise of the mixed-mode column. Therefore, the mixed-mode column Trinity P1 was selected for 3-aminopiperidine analysis.

Optimization of chromatographic conditions for 3-aminopiperidine and linagliptin

Linagliptin is soluble in methanol (approximately 60 mg/mL) and sparingly soluble in ethanol (approximately 10 mg/mL).\textsuperscript{38} So, methanol was preferred as the solvent. Volatile salts such as ammonium formate and ammonium acetate can be used for the CAD. Long \textit{et al.} reported that a better signal-to-noise ratio can be obtained with ammonium formate than with ammonium acetate.\textsuperscript{31} Thus, ammonium formate was used in this study, and its concentration was optimized. Lower concentrations of the buffer solution lead to a smaller background current in the CAD and afforded high sensitivity (Fig. 4). Thus, 10 mM ammonium formate was used as a mobile phase B to separate the target compounds (Fig. 5A). However, 3-aminopiperidine and linagliptin were co-eluted, rendering 10 mM ammonium formate unsuitable for the determination of 3-aminopiperidine in linagliptin samples. In the mixed-mode, three kinds of interactions, namely hydrophobic, cation exchange, and anion exchange interactions, contributing to the retention of ionic compounds.\textsuperscript{4,33} Thus, the retention of ionic compounds could be manipulated by changing the concentration of the organic solvent or buffer solution. It can be seen in Fig. 5B that good
resolution is acquired using 30 mM ammonium formate as a mobile phase B. Good resolution of the target compounds can also be achieved by increasing the initial concentration of the organic solvent. However, the use of acetonitrile was minimized as it was toxic and expensive, and 30 mM ammonium formate was used for the determination of 3-aminopiperidine in linagliptin samples. A higher concentration of buffer solution was not investigated, since the increase in the buffer solution concentration leads to an increase in baseline noise.

Validation of the methods

The quantitative aspects of the proposed methods involving the precision, linearity, limit of detection (LOD), limit of quantification (LOQ), and recovery were examined.

The CAD system generates a parabolic calibration curve and when the concentration is very low (5 - 250 μg mL⁻¹) or it has a narrow range (4 orders of magnitude), the calibration curve is close to being linear for many routine analytical studies.39,40 Seven concentrations of the 3-aminopiperidine sample, ranging from 0.675 to 173 μg mL⁻¹ (i.e., 0.675, 2.73, 5.41, 21.63, 43.25, 86.5, and 173 μg mL⁻¹), were used to obtain calibration curves in this study. The injection volume was 20 μL. The peak areas for three injections of each of the seven standard solutions were plotted versus the concentration of 3-aminopiperidine. Standardization curves of the resulting data were then fitted to a linear trend. The dynamic range of the detector was demonstrated to be 2.73 to 86.5 μg mL⁻¹, with a correlation coefficient (R²) of 0.999. Wide linearity was observed in the current method with CAD. The LOD and LOQ of the current method were 1.35 μg mL⁻¹ (S/N = 6.7) and 2.73 μg mL⁻¹ (S/N = 16.7), respectively. The results indicate that this method is capable of detecting 1.35 μg mL⁻¹ level of 3-aminopiperidine in the drug substance, which is equivalent to about 27 μg of the 3-aminopiperidine per gram of API (270 ppm). The LOD and LOQ of the derivatization method were 0.1 μg mL⁻¹ (S/N = 3.2, 200 ppm) and 0.3 μg mL⁻¹ (S/N = 10.5, 600 ppm), respectively. The sensitivity of the proposed method meets the requirement for the residue detection of 3-aminopiperidine (Table 1).

The recovery of 3-aminopiperidine was evaluated with three concentrations, 4.3 (close to the concentration of LOQ),34 and 51 μg mL⁻¹. As shown in Table 1, the recoveries obtained with these three concentrations are high and overall average recoveries range from 94 to 102%. The recovery of the derivatization method was also investigated (Table 1). Compared to the derivatization method, the current method shows better recovery with various concentrations.

An experiment conducted over two days with six points was performed with a standard solution of 5 μg mL⁻¹ to test repeatability. The raw peak areas were analyzed for a total of 18 unique injections over the two-day study. The inter-day and intra-day results for the peak areas were 0.73 and 1.05%, respectively. The precision of the derivatization method was also investigated (Table 1). Compared to the derivatization method, the current method proved to offer better precision.

The robustness of the method was investigated by deliberately changing the chromatographic conditions. The most important parameter to be studied was the amount of 3-aminopiperidine detected in linagliptin. The flow rate of the mobile phase was changed from 0.7 to 0.6 mL min⁻¹, and 0.8 mL min⁻¹. Additionally, the column temperature was changed from 30 to 25°C and 35°C. Finally, the evaporation temperature of the CAD was set from 55 to 50°C and 60°C. There was no significant difference in the results obtained for any of these variations, indicating good robustness of the proposed LC method (Table 2).

![Fig. 5 Analysis of 3-aminopiperidine and linagliptin with 10 mM ammonium formate (A) and 30 mM ammonium formate (B) as solvent B. Other solvents and gradient conditions have been stated in the chromatographic conditions section.](image)

Table 1 Comparison of results obtained with the proposed method and precolumn derivatization method for 3-aminopiperidine in the linagliptin sample using the charged aerosol and UV detectors

<table>
<thead>
<tr>
<th>Item</th>
<th>Proposed method</th>
<th>Precolumn derivatization method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detector</td>
<td>UV</td>
<td>Chiralpak AD-H (250 × 4.6 mm, 5 μm)</td>
</tr>
<tr>
<td>Column</td>
<td>Acclaim Trinity P1 column (3.0 × 150 mm, 3 μm)</td>
<td>Precolumn derivatization</td>
</tr>
<tr>
<td>Sample preparation</td>
<td>Directly</td>
<td>0.5 mg mL⁻¹ (1% diethylamine methanol)</td>
</tr>
<tr>
<td>Sample concentration</td>
<td>5 mg mL⁻¹ (methanol)</td>
<td>0.3 μg mL⁻¹ (equivalent to 0.05% sample concentration)</td>
</tr>
<tr>
<td>Analysis time</td>
<td>16 min</td>
<td>0.1 μg mL⁻¹ (equivalent to 0.05% sample concentration)</td>
</tr>
<tr>
<td>LOD</td>
<td>2.73 μg mL⁻¹ (equivalent to 0.05% sample concentration)</td>
<td>0.2 - 3.03 μg mL⁻¹</td>
</tr>
<tr>
<td>Linearity</td>
<td>2.73 - 86.5 μg mL⁻¹</td>
<td>1.35 μg mL⁻¹ (equivalent to 0.02% sample concentration)</td>
</tr>
<tr>
<td>Recovery (mean ± SD)</td>
<td>94.19 ± 0.49 (50% identification limit)</td>
<td>93.72 ± 2.44 (50% identification limit)</td>
</tr>
<tr>
<td></td>
<td>99.59 ± 1.25 (100% identification limit),</td>
<td>95.23 ± 1.38 (100% identification limit),</td>
</tr>
<tr>
<td></td>
<td>101.0 ± 1.56 (150% identification limit)</td>
<td>92.6±1.32 (150% identification limit)</td>
</tr>
<tr>
<td>Precisiona</td>
<td>0.73% (inter-day), 1.05% (intra-day)</td>
<td>1.34% (inter-day), 2.28% (intra-day)</td>
</tr>
</tbody>
</table>

a. By RSD of the peak areas (n = 6). b. Threshold of impurity for the structure to be identified within the new drug substance according to the ICH Q3a guideline. c. As reported in reference for 3-aminopiperidine.11
Conclusions

A method utilizing a mixed-mode column combined with a CAD was developed in this study for the determination of 3-aminopiperidine in linagliptin. Chromatographic conditions including the detector, separation mode, and mobile phase composition were optimized. Compared to the precolumn derivatization UV method, the proposed method allowed for more convenient operation, less expense on the column, much shorter analysis time, and a significant decrease in cost and waste generation. Furthermore, a wide range in linearity (2.73 - 86.5 μg mL⁻¹), high sensitivity (LOQ <2.73 μg mL⁻¹), and good precision (RSD of peak area ≤2%) were offered by the method. In addition, the parameters such as buffer solution and column bleed that affected the sensitivity of the CAD were investigated. This is a new non-derivative, highly sensitive, and robust method for the determination of 3-aminopiperidine, constituting a new application of the CAD for the quantitative analysis of highly polar basic compounds.

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Supporting Information

This material is also available online at http://www.jsac.or.jp/analsci/.

References