Identification, Characterization, Synthesis and Quantification of Related Impurities of Liguzinediol

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An HPLC method was employed to create an impurity profile for liguzinediol as an active pharmaceutical ingredient (API), which resulted in the identification of two related impurities. Therefore, in order to improve the quality control of the liguzinediol-API, we identified and then developed a method for quantifying the two impurities (impurity-1 and impurity-2) by LC–TOF–MS–MS and then chemically synthesized them for further studies. Based on spectral data from IR, MS, 1H and 13C NMR, the structures of impurity-1 and impurity-2 were characterized as 2-hydroxymethyl-3,6-dimethylpyrazine and 2-hydroxymethyl-3,5,6-trimethylpyrazine, respectively. We further validated the method according to the International Conference on Harmonization guidelines to demonstrate the sensitivity, precision, linearity, accuracy and stability of the method described. In addition, the potential mechanisms underlying formation of impurity-1 and impurity-2 in the liguzinediol-API are discussed in detail.

Introduction

Ligustrazine, a biologically active alkaloid isolated from the traditional Chinese herb Ligusticum wallichii Franch, has long been used in China to improve circulation and prevent clot formation. It has also shown cardiac effects in animal experiments and suppressed L-type Ca2+ channel currents in rat ventricular myocytes. During a structural modification of ligustrazine, our laboratory discovered liguzinediol (2,5-dihydroxymethyl-3,6-dimethylpyrazine, Figure 1), which is an active derivative of liguaztrazine (1). It has been demonstrated that liguzinediol exerted positive inotropic effects without the risk of arrhythmia by activating the sarcoplasmic reticulum Ca2+ ATPase (SERCA2a) (2–4). Preclinical studies have also shown that liguzinediol shows promise for the treatment of heart failure (5–8).

During our synthetic process (9), liguzinediol is synthesized from 2,5-dimethyl pyrazine via a free radical reaction. Although the purity of liguzinediol-API has been improved up to 99.5% using this process, two related impurities have been detected by HPLC—making it necessary to identify, characterize and quantify the two impurities for comprehensive quality control of liguzinediol-API.

In this study, the AB SCIEX Triple TOF™ 5600 LC–MS–MS system was used to identify the two impurities as 2-hydroxymethyl-3,6-dimethylpyrazine (impurity-1) and 2-hydroxymethyl-3,5,6-trimethylpyrazine (impurity-2), respectively. Subsequently, two synthetic compounds were confirmed to be the chemical equivalents of impurity-1 and impurity-2. We then used the synthetic equivalents to quantify impurities 1 and 2 in the liguzinediol-API, using a Triple Quad™ 5500 LC/MS/MS system.

We confirmed the structure of impurity-1 and impurity-2 using IR, MS, 1H and 13C NMR. In this study, we further define the two impurities identified in the liguzinediol-API synthetic process. Impurity-1 and impurity-2 are speculated to be intermediates or byproducts of the synthetic process of liguzinediol. We also discuss the probable mechanism of their formation.

Experimental

Materials and reagents

Liguzinediol-API (purity >99.5%, HPLC) was synthesized in our laboratory. 2,5-Dimethylpyrazine was obtained from Tengzhou Wutong Aroma Chemicals Co., Ltd. HPLC grade methanol was obtained from Jiangsu Hanbon Sci. & Tech. Co., Ltd. AR grade iron (7) sulfate heptahydrate and sulfuric acid (98%) were obtained from Sinopharm Chemical Reagent Co., Ltd. AR grade tert-butyl hydroperoxide, hydrogen peroxide (30%), sodium hydroxide, petroleum ether and ethyl acetate were all obtained from Nanjing Chemical Reagent Co., Ltd. Ultrapure water was prepared by a Barnstead ultrapure water purification system.

HPLC analysis

A Waters 2695 HPLC system equipped with a 996 photodiode array detector was used for the analysis of the liguzinediol-API. A Phenomenex C18 (250 × 4.6 mm, 5 μm) column was used for the separation. The detection wavelength was set at 278 nm, at which the impurity-1, impurity-2 and liguzinediol showed maximum absorption. A mixture of methanol and water in a ratio of 45:55 was used as the mobile phase at a flow rate of 1.0 mL/min. The injection volume for the sample was 10 μL. The column temperature was maintained at 30°C. Waters Empower 2.0 software was utilized for system control, data collection and analysis.

LC–MS–MS analysis for compound identification

A Shimadzu UFLC LC-20ADXR LC system equipped with an in-line LC-20AD-XR binary pump, SIL-20AC auto sampler and a CTO-20AC column oven was used for chromatographic separation. In addition, a Phenomenex C18 (250 × 4.6 mm, 5 μm) column was used, and the temperature was maintained at 40°C. A mixture of methanol and water with a ratio of 45:55 was used as the mobile phase at a flow rate of 1.0 mL/min. The injection volume for the sample was 2 μL.

For the mass spectrometry analysis, we used an AB SCIEX Triple TOF™ 5600 with a hybrid triple quadrupole time-of-flight mass spectrometer equipped with an electrospray ionization (ESI) source. The mass spectrometer was operated in positive ionization mode for the detection of protonated liguzinediol and its derivatives.
mode with ion spray voltage (ISVF) set at 5,500 V, temperature (TEM) at 500 °C, curtain gas (CUR N₂) at 35 psi, ion source gas 1 (GS1 N₂) at 55 psi and ion source gas 2 (GS2 N₂) at 60 psi. The declustering potential (DP) and collision energies (CE) were set to be 40 and 5 V in MS and 40 and 30 V in MS/MS, respectively. The mass range was set at m/z 50–800. The Peak View™ Software V. 1.2 was used for all the date acquisition and analysis.

LC–MS analysis for compound quantification
An AB SCIEX Triple Quad™ 5500 LC/MS/MS system was interfaced with a Shimadzu UFLC 20ADXR LC system for the simultaneous quantitative (MRM) experiment. The conditions of the LC system were the same as described above. Infusion compound optimization in Analyst® software 1.5.1 was used to optimize the compound-dependent parameters for the impurities of liguazinediol. The mass spectrometer was operated in positive ionization mode with ISVF 5,500 V and TEM of 500 °C. Nitrogen was used as GS1, GS2 and CUR at 50, 50 and 35 AU, respectively. The DP, EP, CE and CXP for impurity-1 and impurity-2 were 55, 10, 19, 12 V and 58, 10, 21, 16 V, respectively. Two MRM transitions 139.1/121.1 (quantifier ion) and 153.0/135.0 ( qualifier ion) were selected for developing the quantitation method for impurity-1 and impurity-2, because these transitions showed better sensitivity and less background noise and interference in comparison with other transitions.

NMR spectroscopy
A Bruker AV-300 instrument was used to collect the NMR spectra of the synthesized compounds. All spectra were acquired in CDCl₃ operating at 300 MHz for ¹H and 75 MHz for ¹³C and the chemical shifts were reported on a δ scale in parts per million relative to tetramethyl silane (δ = 0.00 ppm) as the internal standard. ¹H and ¹³C chemical shifts were also referenced to the residual solvent line at 7.28 and 77.0 ppm, respectively.

IR spectroscopy
The IR spectra for the synthesized compounds were recorded in the solid state as a KBr powder dispersion using a Nicolet Impact 410 (Nicolet Instrument Corp., USA) FT-IR spectrometer.

Synthesis of impurity-1 and impurity-2
These two impurities were synthesized as shown in Figure 2.

**Impurity-1 (2-hydroxymethyl-3,6-dimethylpyrazine)**
2,5-Dimethylpyrazine (4.32 g, 40 mmol) was dissolved in methanol (60 mL, 1,484 mmol), and 30% sulfuric acid (120 mL, 448 mmol) was added at room temperature. Subsequently, 30% hydrogen peroxide (9 mL, 87.9 mmol) and saturated aqueous ferrous sulfate (10.9 g, 39.1 mmol) were added dropwise at the same rate while the reaction temperature was controlled between 30 and 60 °C. The reaction mixture was stirred for an additional 30 min, and then filtered after adjusting to pH = 9. The filtrate was then concentrated in vacuo. The resulting solution was extracted with ethyl acetate (100 mL × 3), and the combined extracts were dried over anhydrous sodium sulfate and filtered. The filtrate was evaporated to oil and purified by the GRACE Reveleris® X2 Flash System to give impurity-1.

**Impurity-2 (2-hydroxymethyl-3,5,6-trimethylpyrazine)**
2,5-Dimethylpyrazine (2.16 g, 20 mmol) was dissolved in methanol (60 mL, 1,484 mmol), and 30% sulfuric acid (90 mL, 335.9 mmol) was added at room temperature. Subsequently, 70% tert-butyl hydroperoxide (14 mL, 98 mmol) and saturated aqueous ferrous sulfate (12.7 g, 45.6 mmol) were added dropwise at the same rate while the reaction temperature was controlled between 30 and 60 °C. The reaction mixture was stirred for an additional 30 min, and then filtered after adjusting to pH = 9. The filtrate was then concentrated in vacuo. The resulting solution was extracted with ethyl acetate (100 mL × 3), and
the combined extracts were dried over anhydrous sodium sulfate and filtered. The filtrate was evaporated to oil and purified by the GRACE Reveleris® X2 Flash System to give impurity-2.

**Purification of impurity-1 and impurity-2 from the synthetic mixture**
The GRACE Reveleris® X2 Flash System was used to purify the synthetic crude product of impurities 1 and 2. A Reveleris® Silica 12 g column was used, and the samples were prepared by mixing with silica (1:1, m/m). The sample detection was monitored at a wavelength of 254 nm (UV1) and 278 nm (UV2), and the UV threshold was set to be 0.05 AU. The collection mode was set to collect the peaks and per-vial volume was 50 mL. The mobile phase for solvent A was petroleum ether and for solvent B was ethyl acetate. A gradient system was employed in the following manner: t (min)/A (v/v)/B (v/v) = 0/75/25, 5/60/40, 10/40/60, 20/10/90, 25/0/100, 30/0/100. The flow rate was set at 10 mL/min.

**Results**

**Detection of impurities by HPLC**
Two impurities in liguzinediol-API were detected by an HPLC method. The impurities were named impurity-1 and impurity-2 in the HPLC chromatogram (Figure 3). The retention times of liguzinediol, impurity-1 and impurity-2 were 3.741, 4.513 and 5.718 min, respectively. The samples of liguzinediol-API were prepared at a concentration of 2 mg/mL in methanol.

**Structure elucidation of liguzinediol and its impurities by LC–MS-MS**
Samples of liguzinediol-API were analyzed by the LC–MS-MS method under positive ESI conditions. Peak View™ Software was used to process the extracted ion chromatograms of liguzinediol and its impurities (Figure 4). As a result, the extracted ion chromatograms were consistent with the HPLC chromatogram for the same sample of liguzinediol-API. The structure elucidation and fragmentation patterns of liguzinediol, impurity-1 and impurity-2 were described as follows.

**Liguzinediol**
The positive ESI-MS spectrum of liguzinediol showed a peak at m/z 169 that was due to [M+H]+. The accurate weight of the molecular ion was m/z 169.0981, and the retention time from the extracted ion chromatograms was 3.715 min. The MS/MS fragmentation of liguzinediol produced fragments of m/z 151.0871, 122.0848 and 121.0768 (Figure 5).

**Impurity-1**
A protonated molecular ion peak was identified at m/z 139 in the spectrum of impurity-1 by the positive ESI-MS. The retention time was 4.501 min, suggesting that impurity-1 had smaller polarity than liguzinediol under these conditions of RP-HPLC. The accurate weight of the molecular ion was m/z 139.0877, corresponding to a possible molecular formula C_7H_10N_2O (calcd 139.0871, [M+H]^+), attributable to the reduction of one hydroxymethyl group of liguzinediol. The MS/MS fragmentation of impurity-1 produced fragments of m/z 121.0773 and 109.0775. The probable fragmentation pattern of impurity-1 is described in Figure 6.

**Impurity-2**
A protonated molecular ion peak was identified at m/z 153 in the spectrum of impurity-2 by the positive ESI-MS. The retention
time was 5.408 min, indicating that impurity-2 had smaller polarity than both liguzinediol and impurity-1. The accurate weight of the molecular ion was \( m/z \) 153.1034, corresponding to a possible molecular formula of \( C_8H_{12}N_2O \) (calcld 153.1028, \([M+H]^+\)) attributable to the reduction of one hydroxyl group of liguzinediol. The MS/MS fragmentation of impurity-2 produced fragments of \( m/z \) 135.0926 and 123.0925. The probable fragmentation pattern of impurity-2 is described in Figure 7.

**Synthesis and structure confirmation of impurity-1 and impurity-2**

The structures of the two impurities were elucidated based on their LC–MS-MS data and were speculated to be intermediates or byproducts of the synthetic process of liguzinediol. Accordingly, the two impurities were synthesized by changing the synthetic process of liguzinediol, yielding 22.1 and 29.3%, respectively. Compound 1 (synthetic impurity-1) was a yellow oil and compound 2 (synthetic impurity-2) was a white crystal. These two synthetic compounds were dissolved in methanol for analysis.

**HPLC analysis of synthetic compounds 1 and 2**

Impurity identification was first conducted by comparing the HPLC retention times and maximum absorption wavelength of synthetic compounds 1 and 2 with those of the impurities (Table 1). As a result, HPLC analysis confirmed that the retention times and maximum absorption wavelengths of synthetic compounds 1 and 2 were identical to their corresponding impurities.

**Mass spectral analysis of synthetic compounds 1 and 2**

The sample solutions of the synthetic compounds were also analyzed by LC–MS-MS to demonstrate equivalence to the corresponding impurities. The retention times for the two synthetic compounds of extracted ion chromatograms were 4.519 and 5.733 min, respectively. The ESI mass spectrum of compound 1 exhibited a protonated molecule peak at \( m/z \) 139.0875 in positive ion mode, and the MS/MS fragmentation produced fragments of \( m/z \) 121.0772 and 121.0775. The ESI mass spectrum of compound 2 exhibited a protonated molecule peak at \( m/z \) 153.1033 in positive ion mode, and the MS/MS fragmentation produced fragments of \( m/z \) 135.0929 and 123.0927 (Figure 8).

Based on the HPLC and LC–MS-MS data, the synthetic compounds were determined to be equivalent to impurities 1 and 2, respectively.

**IR spectroscopy of impurity-1 and impurity-2**

IR spectral data for impurity-1 and impurity-2 are shown in Table II. The IR spectra of both impurity-1 and impurity-2 had
O–H stretching absorption bands of hydroxyl at 3,411 and 3,226 cm$^{-1}$, and the aliphatic C–H stretching absorption bands were at 2,926 and 2,919 cm$^{-1}$. The C–O stretching absorption band of benzylic alcohol is shown at 1,056 and 1,176 cm$^{-1}$. All other data are consistent with the structure of pyrazine.

NMR analysis of impurity-1 and impurity-2
Proton and carbon NMR spectra of liguzinediol and its related impurities are consistent with the structures characterized in this study (Table III). Impurity-1 and impurity-2 were determined to be 2-hydroxymethyl-3,6-dimethylpyrazine and 2-hydroxymethyl-3,5,6-trimethylpyrazine, respectively.

Quantification of impurities
Validation studies for the quantification method of impurity-1 and impurity-2 included sensitivity, precision, linearity, accuracy and stability according to International Conference on Harmonization (ICH) guidelines (10). In the current investigation, impurity-1 and impurity-2 were determined to be 2-hydroxymethyl-3,6-dimethylpyrazine and 2-hydroxymethyl-3,5,6-trimethylpyrazine, respectively.

Sensitivity
Sensitivity was determined by establishing the limit of detection (LOD) and limit of quantification (LOQ) for impurity-1 and impurity-2 by injecting a series of diluted solutions at known concentrations. The signal-to-noise ratios are presented as 3:1 for LOD and 10:1 for LOQ, which were measured by Analyst® software. The LOD values are 0.10 ng for impurity-1 and 0.02 ng for impurity-2. The LOQ values are 0.30 ng for impurity-1 and 0.04 ng for impurity-2, respectively (Figure 9).

Precision
For system precision measurements, mixed standard solutions of impurity-1 and impurity-2 at 400, 600 and 800 ng/mL were injected in triplicate for each concentration under the same conditions to check the relative standard deviation (RSD). The RSD

Table I

<table>
<thead>
<tr>
<th>Samples</th>
<th>HPLC retention times (min)</th>
<th>Maximum absorption wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impurity</td>
<td>4.51 5.72</td>
<td>277.0 279.3</td>
</tr>
<tr>
<td>Compound</td>
<td>4.50 5.72</td>
<td>277.2 279.4</td>
</tr>
</tbody>
</table>


for impurity-1 was 1.88%, and for impurity-2 was 0.93%. These data are all within the generally acceptable limit of 3% (Table IV).

Linearity
To examine linearity, impurity-1 and impurity-2 were diluted to prepare a series of mixed standard solutions of 100, 200, 400, 600 and 800 ng/mL. Good calibration curves for each impurity standard solution were obtained. The regression equations for impurity-1 and impurity-2 are $Y = 20.710X + 402.398$ ($R^2 = 0.9995$) and $Y = 39.974X + 2E + 06$ ($R^2 = 0.9992$), respectively. The calibration curves were proven to be linear in the range of 100–800 ng/mL for both impurity-1 and impurity-2 (Figure 10).

Accuracy
System accuracy was assessed for the related substances by spiking of known amounts of an impurity in samples of liguzinediol-API (test preparation) at levels of 80, 100 and 120% of the specified limit. The recovery of impurities was calculated from the following equation:

$$R(a)\% = \frac{C_{\text{obs}(a)} \text{ in spiked sample} - C_{\text{obs}(a)} \text{ in unspiked sample}}{C_{\text{spike}(a)}} \times 100\%.$$

Mean recovery for impurity-1 and impurity-2 at the 80% level was calculated as 103.47 and 99.09%; at the 100% level, recovery was 101.26 and 101.61%; and at the 120% level, recovery was 100.40%.
and 100.59%, respectively. The results suggest that the assay was satisfactory with a mean recovery from 99.09 to 103.47% and an RSD <1.45% for the mean recovery.

**Stability**
Stability of both the standard solution of liguzinediol and mixed standard solutions of impurity-1 and impurity-2 was determined at 0, 2, 4, 6, 8, 10, 12, 24, 48 and 72 h against freshly prepared solutions. The stability of the solutions was evaluated by leaving the samples in tightly capped HPLC vials for 72 h at 30°C in an autosampler. The RSD of experimental data for solution stability was calculated and confirmed that sample solutions were stable up to 72 h, and the retention times of these three compounds remain stable for 72 h.

**Quantification**
The developed method was applied for the quantification of the two impurities in liguzinediol-API defined in this study. A liguzinediol concentration of 200 μg/mL was selected for these studies to ensure that the concentrations of impurity-1 and impurity-2 were within the corresponding calibration curves. The results show that the content of each impurity was within the detection limit according to the ICH guidelines (Table V). Furthermore, the liguzinediol purification process in our laboratory was feasible and stable. The quality of our synthesized liguzinediol was consistent with the standards for pharmaceutical ingredients.

**Discussion**
The results from these experiments show that the two impurities of liguzinediol can be easily detected by HPLC, but not accurately quantified. Consequently, it was necessary to develop a sensitive method for the quantification of liguzinediol impurities by LC-MS. The methods proposed in this study are optimal for the analysis of liguzinediol and its impurities.

In Figure 11, we suggest a probable formation mechanism of liguzinediol impurities. The hydroxyl radical, which was generated via the reaction between H₂O₂ and Fe²⁺+, was reacted with methanol to afford a hydroxymethyl radical. The three-position of the pyrazine ring was then attacked by the hydroxymethyl radical to give impurity-1. Impurity-1 was then reacted with another hydroxymethyl radical to give liguzinediol. Therefore, we suggest that impurity-1 is an intermediate of the synthetic process of liguzinediol. Reacting impurity-1 with another methyl radical yields impurity-2, which is suggested to be a byproduct of the synthetic process. The formation of impurity-1 might be caused by the steric hindrance of the substituent group on the pyrazine ring. It was also found that the concentration of the hydroxymethyl radical in the reaction mixture might have an influence on the formation of impurity-1. Similarly, we could change the proportion of H₂O₂, Fe²⁺+, and CH₃OH to obtain impurity-1.

The amount of CH₃OH used in the synthesis of liguzinediol has a variable impact on yield. Amount in the range of 30 mL is

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**Table II**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IR Spectral Data for Liguzinediol, Impurity-1 and Impurity-2 (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liguzinediol</td>
<td>3,387 (O–H stretching), 2,739 (methyl C–H stretching), 2,618 (methylenes C–H stretching), 1,476 (C= C stretching), 1,465 (C=N stretching), 1,426 (methylene C–H bending), 1,366 (methyl C–H bending) and 1,172 (C–O stretching)</td>
</tr>
<tr>
<td>Impurity-1</td>
<td>3,411 (O–H stretching), 2,926 (aliphatic C–H stretching), 1,459 (C=C stretching), 1,378 (methylene C–H bending), 1,582 (methyl C–H bending) and 1,056 (C–O stretching)</td>
</tr>
<tr>
<td>Impurity-2</td>
<td>3,226 (O–H stretching), 2,919 (aliphatic C–H stretching), 1,526 (C=C stretching), 1,518 (C=N stretching), 1,412 (methylene C–H bending), 1,312 (methyl C–H bending) and 1,176 (C–O stretching)</td>
</tr>
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positively correlated with the yield of liguzinediol, whereas amount over 30 mL is negatively correlated with yield (Figure 12A). This observation may be a result of CH₃OH's ability to serve as both a radical donor and solvent. Therefore, to a
In certain extent, the CH₃OH determines the concentration of the hydroxymethyl radical generated. However, when the volume of CH₃OH is sufficiently large enough to dilute the reaction solution, then production of the hydroxymethyl radical is reduced—along with decreasing yields of both liguzinediol and impurity-1. In addition, the amount of H₂O₂ is positively correlated with the yield of liguzinediol and negatively correlated with the yield of impurity-1 (Figure 12B). This is likely due to a positive correlation between H₂O₂ and the concentration of hydroxymethyl radical, with impurity-1 as the intermediate product of the reaction. In summary, the production of the hydroxymethyl radical determines, in large part, the percentage of impurity-1 that is generated during the production of liguzinediol-API.

The proportion of H₂O₂, Fe²⁺, and CH₃OH showed no influence on the yield of impurity-2; however, we found that the yield of impurity-2 could be increased significantly by using tert-butyl hydroperoxide instead of H₂O₂. This is likely due to an attack by a methyl radical on the 6-position of impurity-1 in the presence of tert-butyl hydroperoxide when compared with H₂O₂. Because tert-butyl hydroperoxide produced more methyl radicals during the reaction than H₂O₂, we chose it as the reagent to synthesize impurity-2.

**Conclusion**

In this study, two process-related impurities of liguzinediol-API were identified, characterized and chemically synthesized for the first time using LC–MS-MS. The preparation was controllable and convenient, and the detection methods were reliable, reproducible and specific. Further study of the synthesis process involved in the two impurities will be beneficial for pilot-scale production of liguzinediol.

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**References**


