Isolation and Identification of a Major Impurity in a New Bulk Drug Candidate by Preparative LC, ESI-MS^n, LC–MS–MS, and NMR

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Abstract

A simple preparative liquid chromatography (LC) method is developed to isolate a major impurity in a new bulk drug candidate, 6-bromo-4-(carbamidinemethyl)-5-hydroxy-1-methyl-2-(phenylthiomethyl)-1*H*-indole-3-carboxylic acid ethyl ester hydrochloride monohydrate (carmidole). The carmidole solution for preparation is exposed to daylight before isolation. Based on the electrospray ionization (ESI)–mass spectroscopy (MS^n) spectral data of the impurity fraction and carmidole, the impurity is preliminarily characterized as 6-bromo-4-(carbamidinemethyl)-5-hydroxy-1-methyl-2-methyl-1*H*-indole-3-carboxylic acid ethyl ester. LC–MS–MS is used to analyze a carmidole sample. The impurity, lyophilate, is obtained from the fraction of preparative LC, and the impurity standard is synthesized. By comparison of the retention times of high-performance liquid chromatography, ESI-MS^n, and ^{1}H-nuclear magnetic resonance of the impurity lyophilate with impurity standard and carmidole itself, the structure of the impurity is confirmed and its formation is discussed.

Introduction

6-Bromo-4-(carbamidinemethyl)-5-hydroxy-1-methyl-2-(phenylthiomethyl)-1*H*-indole-3-carbox-ylic acid ethyl ester hydrochloride monohydrate (carmidole) is a bulk drug candidate with a new structure (Figure 1). Pharmacodynamical investigation revealed that in vitro it could notably inhibit the cytophathic effect of the influenza virus, respiratory syncytial virus, adenovirus III, parainfluenza virus, and herpes simplex virus I; whereas in vivo it could effectively restrain the virus pneumonia (arisen from the influenza virus) in mice. Carmidole is being comprehensively investigated in order to develop it into a new antiviral drug, and the present report mainly discusses the identification of a major impurity in carmidole.

In recent years, there has been an increased awareness of the importance of characterizing and determining impurities in new drug substances being developed. According to a guideline entitled *Impurities in New Drug Substances* by the International Conference on Harmonization (ICH) (1), all impurities at a level of 0.1% or greater in a new drug substance should be isolated and identified. Isolating and identifying impurities serves two

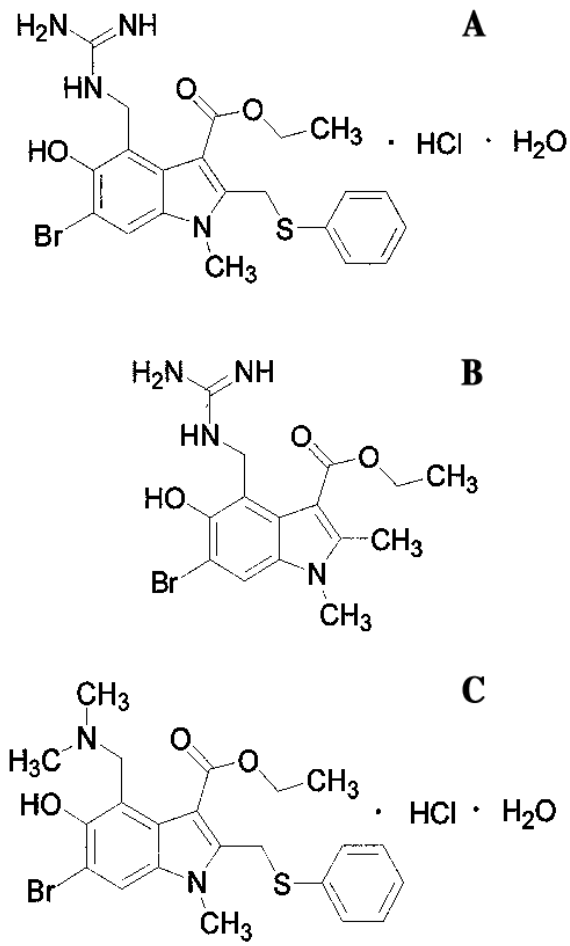

Figure 1. Structures of carmidole (A), impurity (B), and arbidol (C).

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purposes. First, identification can help determine the origin of the impurity, allowing the synthesis to be modified to reduce the percentage of the impurity in the final product. Second, isolation and identification allows the level in the final product to be accurately determined. The ICH guideline also enumerates that potential and actual impurities may arise from starting material and its impurities, by-products, intermediates, degradation products, reagents, ligands, and catalysts (2). In fact, the impurities are mostly unknown and present at a very low level; therefore, it is difficult to isolate and identify them.

Two methodologies can be used for impurity identification. One is analytical separation coupled with spectroscopic identification, such as gas chromatography (GC)–mass spectroscopy (MS) (3), liquid chromatography (LC)–MS (4–6), and LC–nuclear magnetic resonance (NMR) (7–9). The other is preparative LC to isolate the low-level impurities and identify the isolated material by spectroscopic techniques (10–14). When impurities are simple analogues of a drug substance, their structures may be concluded with online spectroscopy. Unless conclusive information can be obtained, the unknown impurities need to be isolated and identified by spectroscopic techniques. For complete verification, it may be necessary to synthesize the impurity standard and compare its spectroscopic characteristics with those observed in the original sample (15).

In this study, a major impurity in carmidole is successfully isolated and identified by using preparative LC, electrospray ionization (ESI)-MS^n, LC–MS–MS, and NMR. To the best of our knowledge, carmidole and its impurity have not yet been reported in the literature.

Experimental

Preparative LC system

An Elite HPLC equipped with a P260 pump and UV200 II detector (Dalian Elite Analytical Instruments, Dalian, China) and a Rheodyne Injector model 3725i (Rheodyne, Cotati, CA) with a 10.0-mL loop were used. A Sinochrom ODS column (250 × 20-mm, 15 µm, Elite Analytical Instruments) was used for isolation, and the mobile phase consisted of water and methanol in the ratio of 40:60 (v/v). The flow rate was 10.0 mL/min, the detector wavelength was 254 nm, the injection volume was 10 mL, and the column temperature was ambient. The data was recorded using an Anastar chromatographic workstation (Tianjin Autoscience, Tianjin, China).

ESI-MS^n

An Agilent 1100 Series SL ion trap MS (Agilent Technologies, Palo Alto, CA) equipped with an ESI probe was operated in positive ion mode. The operating parameters for MS analysis were: nebulizer nitrogen gas pressure at 0.12 Mpa (15 psi) and a drying nitrogen gas rate at 5 L/min. The drying gas temperature was 300°C. The scan range was 50–800 amu.

LC–MS–MS system

A Finnigan LCQ ion trap MS (Thermo Finnigan, San Jose, CA) equipped with an ESI probe was operated in positive ion mode. A Diamonsil C_{18} column (250 × 4.6 mm, 5 µm, Dikma Technologies, Beijing, China) was used for the separation at room temperature, and the mobile phase consisted of 0.01M NH_4Ac (pH 6.0 with NH_4OH) and methanol in the ratio of 30:70 (v/v). The flow rate was 0.5 mL/min, and 20 µL was injected. The operating parameters for online LC–MS–MS analysis were: capillary temperature, 200°C; capillary voltage, 25 kV; sheath gas (N_2) flow rate, 50 a.u.; auxiliary gas flow rate, 10 a.u.; and scan range, 50–1000 amu.

^{1}H-NMR spectroscopy

^{1}H-NMR spectra were obtained with a Bruker ARX-300 spectrometer (Bruker, Faellanden, Switzerland), set to the following parameters: 300 MHz at 25.0°C; data point resolution, 0.332 Hz per point; decoupling frequency, 5435; pulse width, 8 µs; relaxation delay, 4 s; acquisition time, 1.507 s; and number of scans, 32.

Samples and reagents

The investigated samples were synthesized by Professor Ping Gong's laboratory of Shenyang Pharmaceutical University (Shenyang, China). The impurity standard was synthesized from the same laboratory. LC-grade solvents and distilled water were used for all mobile phases. HPLC-grade methanol was purchased from Shandong Yuwang Chemical Factory (Shandong, China). Dimethyl-d_6 sulfoxide (DMSO), isotopic purity > 99.9 atom%, was obtained from Aldrich Chemical (Milwaukee, WI).

Results and Discussion

Isolation and characterization of the major impurity

In several batches of carmidole, the majority of impurities were controlled at low levels and did not require identification. However, the major impurity, present nearly at a level of 0.1%, resisted identification. Direct LC–MS was used first, but the concentration of the impurity was too low to generate structural information. Therefore, it was necessary to isolate the impurity for identification.

The level of impurity in crystallization mother liquor was

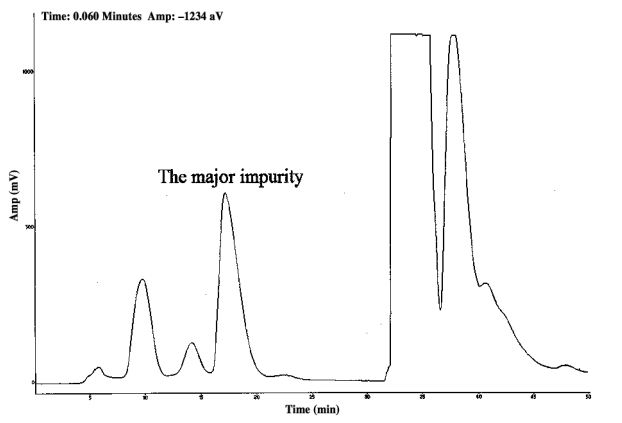

Figure 2. Chromatogram of carmidole solution under light for 2 h at 10 mg/mL concentration under preparative LC condition.

not much higher than that in the drug, and there were some other impurities interfering with the isolation. For example, the mother liquor was not considered useful as a starting source of the impurity, and the finished bulk drug substance was used as the source of impurity. Based on the phenomenon that the major impurity increased in the solution of carmidole under daylight, carmidole solution was exposed to daylight for 2 h before isolation. Even though the photochemical reaction had been used, a much lower level of the impurity than carmidole existed in the solution; therefore, thin-layer chromatography (TLC) and solid-phase extraction (SPE) were not successful in enriching or purifying the impurity because of the interference of carmidole. A simple preparative LC was then developed for the isolation. The chromatogram is shown in Figure 2.

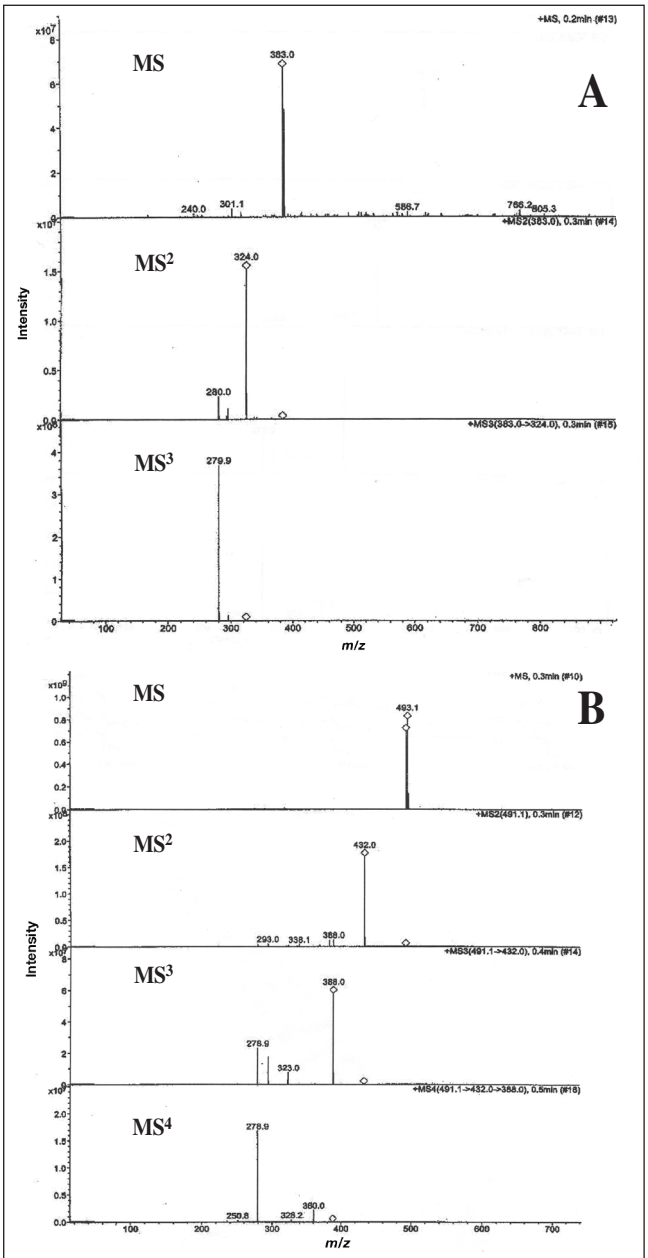

Figure 3. ESI-MS^n spectra of impurity fraction (A) and carmidole (B).

The impurity fraction and carmidole were analyzed by ESI-MS^n. The spectra are presented in Figure 3. Both mass spectra displayed two peaks, which are the protonated species $[M+H]^+$ and $[M+H+2]^+$. Their ratio of relative absorbance was 1:1, indicating a single bromine being present. By comparison, it was found that the molecular weight of the impurity was 108 amu less than that of carmidole. In the collision-induced decomposition process, the protonated ion of impurity lost two neutrals (*m/z* 59 amu and 44 amu) and the protonated ion of carmidole lost three neutrals (*m/z* 59 amu, 44 amu, and 108 amu). Thus, it can be seen that the structures of impurity and carmidole should have the same parent group, and the difference between them lies in the neutral of *m/z* 108 amu. According to the structure of carmidole, it can be deduced that the neutral of *m/z* 108 amu may be a thiophenol group, the neutral of *m/z* 59 amu may be derived from a carbamidine group, and the neutral of *m/z* 44 amu may come from acetaldehyde. The fragmentation pathway of the ion at *m/z* 383 amu is present in Figure 4. Based on these deductions, the impurity was characterized as 6-bromo-4-(carbamidinemethyl)-5-hydroxy-1-methyl-2-methyl-1*H*-indole-3-carboxylic acid ethyl ester (Figure 1). The comparison of the ESI-MS^n spectra of an impurity fraction with carmidole was the key to identification of the impurity.

Figure 5A is the total ion chromatogram (TIC) of a carmidole sample under the LC–MS–MS condition. It could be observed from an enlarged TIC chromatogram that a reasonable separation between the major impurity and other peaks has been achieved. Then, selected ion monitoring (SIM) was used, and the ion at *m/z* 383 amu was monitored in a LC–MS–MS analysis. The SIM chromatogram and two full MS^2 spectra of an impurity from the carmidole sample are shown in Figures 5B, 5C, and 5D. The molecular ions and fragment ions of an impurity directly from the carmidole sample and an impurity fraction from the preparative LC were identical, indicating that the impurity in the solution for the preparation had not changed during the course of sample treatment. Afterwards, the major impurity was isolated by fraction collection at 17–18 min by preparative

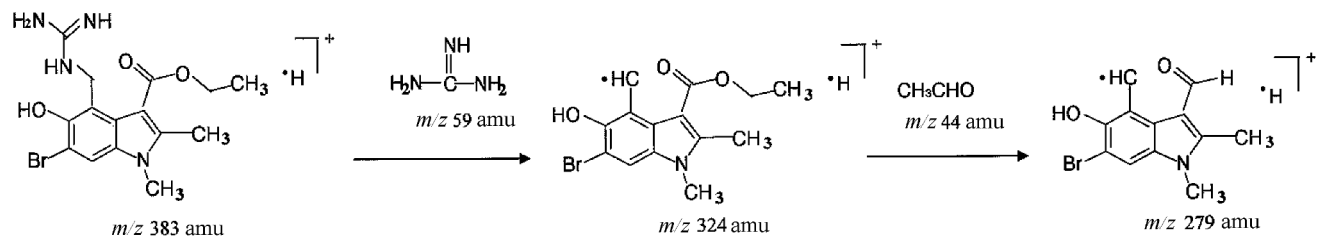

Figure 4. Fragmentation pathway of the ion at *m/z* 383 amu.

LC. The column was washed with methanol between injections. All the fractions were concentrated under a vacuum on a rotary evaporator to strip off the organic solvent. The remaining aqueous layer was removed by freeze-drying to give a white lyophilate. The HPLC purity of the major impurity was 93% (area percent).

Structural elucidation and formation of impurity

^{1}H- and ^{13}C-NMR is a powerful, unequivocal technique in the structure characterization of a compound. The isolated impurity was subjected to an NMR analysis; however, no ^{13}C-NMR spectra data was obtained because the impurity isolated from the preparative LC had very poor solubility in solvents used for the NMR analysis. Thus, impurity standard was synthesized on the basis of the previously described characterization. ^{1}H-NMR assignments of impurity lyophilate and impurity standard are listed in Table I and are compared with each other. They were identical, and the attachment caused by the thiophenol group, as in carmidole (Table II), was not observed, which confirmed deductions based on the ESI-MS^n spectra. Furthermore, the synthesized impurity standard HPLC retention time matched well with the impurity present in carmidole. Comparisons of the retention time (HPLC), ESI-MS^n, and ^{1}H-NMR of the impurity lyophilate with the impurity standard support the characterization of this impurity as 6-bromo-4-(carbamidinemethyl)-5-hydroxy-1-methyl-2-methyl-1*H*-indole-3-carboxylic acid ethyl ester.

The major impurity was formed through three routes. First, 6-bromo-4-(dimethylamino- methyl)-5-hydroxy-1-methyl-2-(phenylthiomethyl)-1*H*-indole-3-carboxylic acid ethyl ester hydrochloride monohydrate (arbidol, see Figure 1), an immediate precursor of carmidole, may have lost the thiophenol group during the course of storage, causing the major impurity to be produced in the following synthesis step. Second, and most important, the carmidole in the solution transformed into the impurity when exposed to light in the last step of synthesis. Therefore, this indicated that synthesis should be performed under dim light. Finally, the impurity also may be a degradation product during storage or under stress conditions.

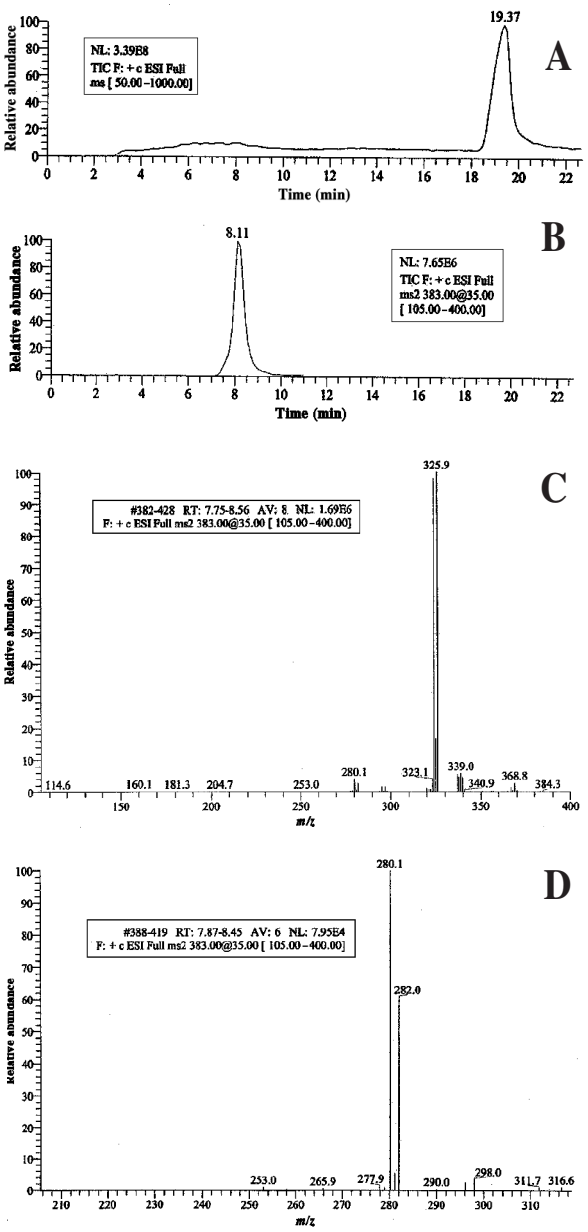

Figure 5. The TIC of carmidole sample (A); SIM chromatogram (B); and two full MS^2 spectra of impurity form carmidole sample (C and D) under LC–MS–MS condition.

Table I. ^{1}H-NMR Assignments of Impurity Lyophilate and Impurity Standard

No.	δ (ppm) Impurity lyophilate	δ (ppm) Impurity standard	Number of H	Attachment
1	1.34	1.34	3,t,J_{A2X3} = 7.1Hz	–OCH_2CH_3
2	2.56	2.55	3, s	–CH_3
3	3.56	3.55	3, s	N-CH_3
4	4.26	4.28	2, q	–OCH_2CH_3
5	4.58	4.55	2, d	–NH-CH_2
6	6.65	6.57	2, s	–NH_2
7	6.95	6.91	1, t	–CH_2-NH-
8	7.47	7.43	1, s	–ϕH

Table II. ^{1}H-NMR Assignments of Carmidole

No.	δ (ppm)	Number of H	Attachment
1	1.23	3,t,J_{A2X3} = 7.2 Hz	–OCH_2CH_3
2	3.51	3, s	N-CH_3
3	4.17	2,q,J_{A2X3} = 7.2 HZ	–OCH_2CH_3
4	4.55	2, d	–NH-CH_2
5	4.65	2, s	–S-CH_2
6	6.62	2, s	–NH_2
7	6.93	1, t	–CH_2-NH-
8*	7.28–7.39	5, m	–ϕH
9	7.48	1, s	–ϕH

* Attachement caused by thiophenol group.

Conclusion

A simple preparative LC method was developed, and a particular sample treatment procedure was used. Identification of the impurity as 6-bromo-4-(carbamidinemethyl)-5-hydroxy-1-methyl-2-methyl-1*H*-indole-3-carboxylic acid ethyl ester was accomplished by a combination of ESI-MS^n, LC–MS–MS, and NMR.

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