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Characterization of impurities in semi-synthetic vinorelbine bitartrate by HPLC-MS with mass spectrometric shift technique

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Abstract

A simple and sensitive method of high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC/ESI-MS) was developed to separate and identify impurities in semi-synthetic vinorelbine bitartrate sample. The analytical HPLC was carried out on a reversed-phase C8 column using 0.02 M ammonium formicate buffer (pH 4.2) and methanol (46:54, v/v) as mobile phase at a flow rate of 0.8 ml/min at room temperature and a UV detection at 267 nm. The on-line HPLC/ESI-MS/MS was performed using ion trap analyzer in positive ion mode. Applying mass spectrometric shift technique to HPLC/ESI-MS/MS analysis, four impurities were identified as 18′-*O*-demethylvinorelbine (impurity-1), 6′-*N*-methylvinorelbine (impurity-2), 23-*O*-demethylvinoreline (impurity-3) and 17-bromovinorelbine (impurity-4), respectively, in investigated vinorelbine bitartrate sample. The four impurities, in which the impurity-1 was not reported as the semi-synthetic process impurity for vinorelbine bitartrate elsewhere, were isolated by preparative high-performance liquid chromatography. Their structures were further confirmed by means of 1D and 2D NMR spectra. Structural elucidation by spectral data was discussed.

Keywords: Vinorelbine bitartrate; Mass spectrometric shift technique; HPLC/ESI-MS/MS; Structure elucidation

1. Introduction

Vinorlbine bitartrate, marked under the name Navelbine, is a unique semi-synthetic vinca alkaloid, in which the catharanthine moiety contains an eight-membered ring in place of nine-membered ring that is present in all naturally occurring members of the vinblastine group. Vinorelbine, first prepared and characterized in 1979 by Potier and co-workers [1], has been demonstrated significant clinical activities against non-small cell lung cancer, bronchial, adenocarcinoma, breast cancer, and head/neck squamous cell carcinoma. Moreover, it has been widely employed in combination with cisplatinum with or without 5-fluorouracil for the treatment of lung cancer and head/neck carcinomas [2,3]. Although its mechanism of action acts as that of vinblastine group, vinorelbine, with improved antitumor activity, has lower neurotoxicity than vinblastine group.

In spite of the attention directed towards the analysis of vinorelbine in biological fluids by HPLC and other methods [4–10], there is scarcely report on method of high-performance liquid chromatography coupled with mass spectrometry for impurities detection in the bulk drug of vinorelbine bitartrate. It is a stringent regulatory requirement that all the impurities $\geq$0.1% must be identified and characterized [11]. The purpose of this study was to develop a simple and sensitive high-performance liquid chromatography coupled with mass spectrometry method for identification and characterization of potential impurities at level of <0.1% in the bulk drug of vinorelbine bitartrate. The structures of impurities were also confirmed by means of 1D and 2D NMR spectra.

2. Experimental

2.1. Chemical and reagents

The investigated samples were kindly supplied by Hisor. Pharm. Inc., Hangzhou, China. Methanol used for analytical

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and preparative HPLC was of chromatographic grade and purchased from Merck, Darmstadt, Germany. Deionized water (18 MΩ) was obtained from a Milli-Q purification system, Millipore, Bedford, MA, USA. Ammonium formicate solids were reagent grade, purchased from Xudong Chemical Plant, Beijing, China.

2.2. High-performance liquid chromatography (analytical)

The analytical HPLC was performed using an Agilent 1100 chromatography system including a G1312 Binpump, G1314A variable-wavelength detector (VWD), model 7725 injector fitted with 20 μl sample loop, and an Agilent ChemStation for LC. A reversed-phase Eclipse XDB-C8 column (Zorbax, 150 mm × 4.6 mm i.d., 5 μm) was used for separation. The mobile phase was 0.02 M ammonium formicate (adjusted to pH 4.2 with formic acid) and methanol (46:54, v/v). The flow rate was 0.8 ml/min, and the effluent was monitored at 267 nm.

2.3. High-performance liquid chromatography (preparative)

An Agilent 1100 preparative chromatography system was used. The preparative separation was achieved on a reversed-phase column (Hypersil C18, 250 mm × 20.0 mm i.d., 5 μm) using the same mobile phase as described in Section 2.2 at a flow rate of 28 ml/min at room temperature, and an UV detection at 267 nm.

2.4. Mass spectrometry

The HPLC system described in Section 2.2 was online coupled to a Bruker Esquire 3000^{plus} mass spectrometer (Bruker-Franzen Analytik GmbH Breman, Germany) equipped with ESI interface and ion trap analyzer. Instrument control and data acquisition were performed using software Esquire 5.0. The ion source temperature was 250 °C, and the source voltage was always set at 3.5 kV. Nitrogen was used as the sheath and nebulizer gas with flow rate at 8 l/min and pressure at 25 psi, respectively. Helium was applied to the system with an estimated pressure of 6×10^{-6} mbar to improve trapping efficiency, and also provided as the collision gas during the tandem mass experiment. For MS/MS spectra, the mass spectrometer was tuned optimizing the specific collision energy 0.65 V to maximize the ion current of sequential MS/MS of vinorelbine and four impurities.

The high-resolution mass spectrum was obtained on a Bruker 7-tesla FT-ICR MS equipped with an Analytica electrospray source (Bradford, CT). The nebulizing and drying nitrogen pressure were maintained at 50 and 30 psi, respectively. All the data were acquired and processed using Bruker Xmass Version 6.1.1 software (Billerica, MA).

2.5. NMR spectroscopy

^{1}H and ^{13}C NMR spectra were measured on a Bruker Advance DMX 500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, using DMSO-d_6 or $CDCl_3$ as solvent. The chemical shifts were referenced to solvent DMSO-d_6 or $CDCl_3$. The experiments were performed at 298 K. 1D ^{1}H and ^{13}C NMR spectra were acquired under standard conditions (5 mm QNP probe), and the 2D experiments were acquired and processed with XWINNMR Version 2.1 software (Bruker Analytik GmbH, Germany). ^{1}H–^{1}H COSY spectra were obtained with 1024 × 256 data points, relaxation delay 1.5 s. The one-bond hetero-nuclear correlation (HMQC) experiments were carried out with 1024 × 256 data points, relaxation delay 2.5 s. The long-range ^{1}H–^{13}C correlation (HMBC) spectra were obtained with 1024 × 256 data points, relaxation delay 2.0 s.

3. Results and discussion

3.1. Detection and isolation of impurities 1–4

A typical HPLC spectrum for the bulk drug of vinorelbine bitartrate was shown in Fig. 1, the contents of impurities are all at level of <0.1%. The signal at retention time of 1.80 min is bitartrate. The target impurities recorded at retention time of 4.59, 8.94, 10.74, and 30.34 min, respectively, were marked as impurity-1, impurity-2, impurity-3, and impurity-4. An isocratic reverse phased solvent system

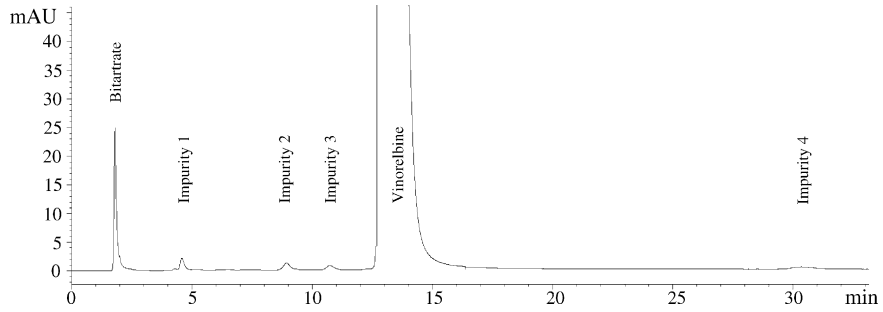

Fig. 1. HPLC chromatogram of the vinorelbine bitartrate bulk drug. For the chromatographic conditions see Section 2.2.

discussed under Section 2.3 was used for the separation of these impurities. All the fractions of impurities isolated were concentrated and extracted with chloroform. The isolated solids obtained from the concentrated fractions of impurities were used to generate spectral data. The details of the elucidation of structures for these impurities were presented in the following sections. The structures of vinorelbine and impurities were listed in Fig. 2.

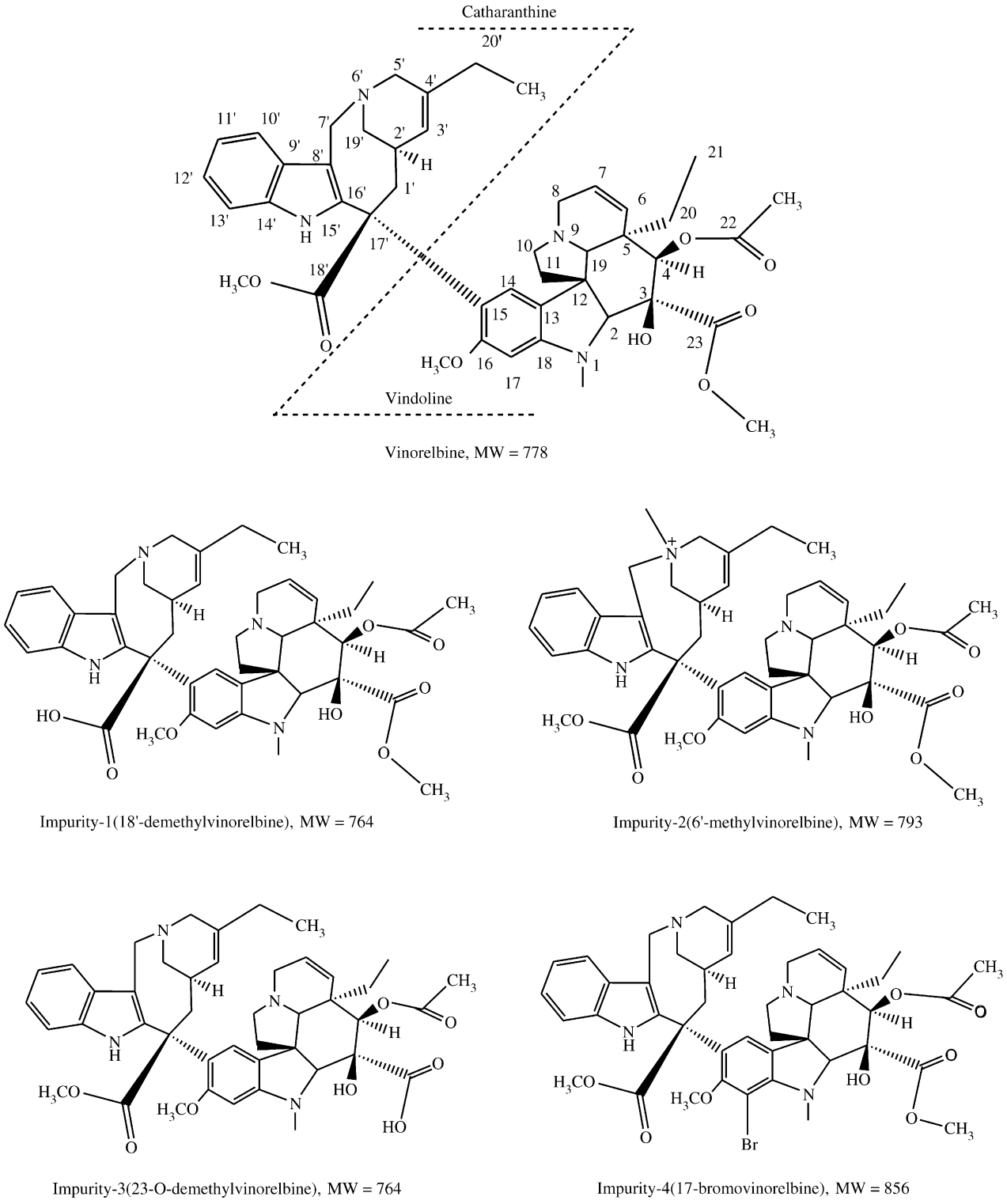

Fig. 2. Structures of vinorelbine and its related impurities.

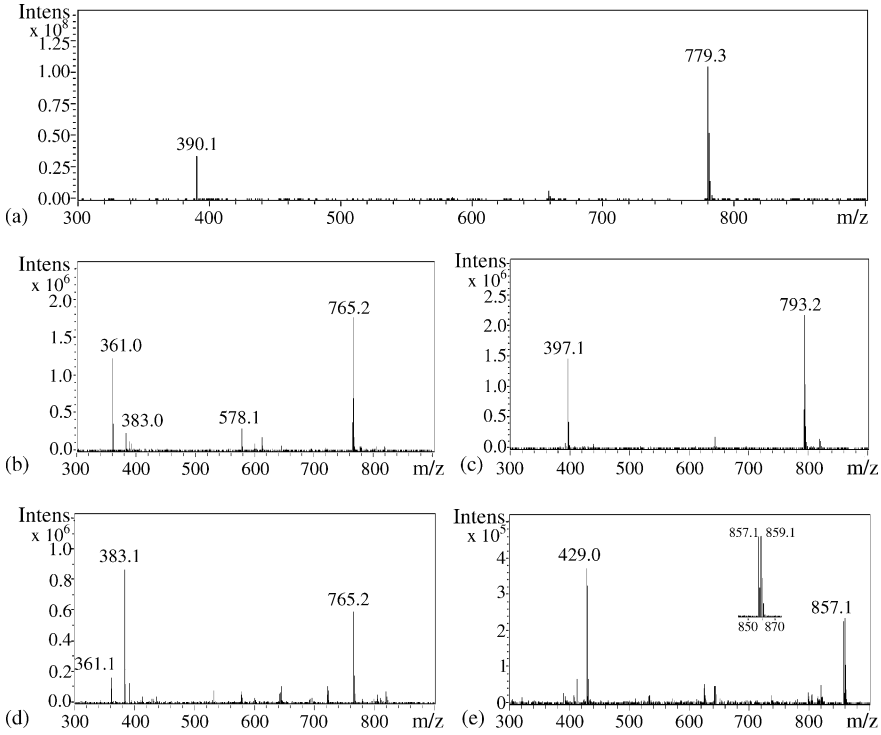

Fig. 3. Positive ion mode HPLC/ESI-MS analyses of vinorelbine, impurity-1, impurity-2, impurity-3 and impurity-4, (a) ESI-MS of vinorelbine; (b) ESI-MS of impurity-1; (c) ESI-MS of impurity-2; (d) ESI-MS of impurity-3; (e) ESI-MS of impurity-4, in which the insert shows the expanded view for *m*/*z* 850–870.

3.2. Structural elucidation

Vinorelbine is a bis-indole alkaloid, which consists of catharanthine moiety and vindoline moiety. The protonated molecular ion $[M+H]^+$ of vinorelbine was recorded at *m*/*z* 779 in the on-line positive HPLC/ESI-MS spectrum (Fig. 3a). The positive ion mode HPLC/ESI-MS/MS (Fig. 4a) scan on the parent ion *m*/*z* 779 yielded characteristic fragment ions at *m*/*z* 719 $[M+H–HCOOCH_3]^+$, 658 $[M+H–C_8H_{11}N]^+$, 457 (vindoline fragment pattern) and 323 (catharanthine fragment pattern). The 1D and 2D NMR data of investigated vinorelbine are well in agreement with that of published in literature [12].

The partial data of ^1H and ^{13}C NMR for vinorelbine and related impurities were listed in Tables 1 and 2, respectively.

Table 1
^1H NMR data of vinorelbine and impurities

Atom	Vinorelbine, δ (ppm)	Impurity-1, δ (ppm)	Impurity-2, δ (ppm)	Impurity-3, δ (ppm)	Impurity-4, δ (ppm)
17	6.40(1H,s)	6.35(1H,s)	6.41(1H,s)	6.39(1H,s)	–
23-$COOCH_3$	3.64(3H,s)	3.64(3H,s)	3.64(3H,s)	–	3.58(3H,s)
5′	3.92(1H,d), 3.58(1H,d)	3.64(1H,d), 3.17(1H,d)	4.13(2H,m)	3.96(1H,d), 3.76(1H,d)	4.00(1H,d), 3.55(1H,d)
7′	4.82(1H,d), 4.42(1H,d)	4.65(1H,d), 4.34(1H,d)	4.87(2H,m)	4.90(1H,d), 4.50(1H,d)	4.86(1H,d), 4.43(1H,d)
19′	3.55(1H,d), 2.68(1H,d)	3.43(1H,d), 2.61(1H,d)	3.44(1H,d), 2.98(1H,d)	3.61(1H,d), 2.78(1H,d)	3.55(1H,d), 2.70(1H,d)
18′-$COOCH_3$	3.68(3H,s)	–	3.68(3H,s)	3.67(3H,s)	3.72(3H,s)
6′-NCH_3	–	–	3.02(3H,s)	–	–

Table 2
^{13}C NMR data of vinorelbine and impurities

Atom	Vinorelbine, δ (ppm)	Impurity-1, δ (ppm)	Impurity-2, δ (ppm)	Impurity-3, δ (ppm)	Impurity-4, δ (ppm)
17	94.3	94.9	93.9	93.6	100.8
23	170.5	170.4	171.1	172.0	170.7
23-$COOCH_3$	52.1	52.0	51.6	–	52.1
5′	52.2	52.9	61.7	51.7	52.3
7′	46.7	46.9	54.8	46.4	46.2
19′	43.5	43.9	53.1	43.4	43.2
18′	174.0	175.6	173.2	173.4	173.4
18′-$COOCH_3$	53.2	–	52.9	52.7	53.4
6′-NCH_3	–	–	51.6	–	–

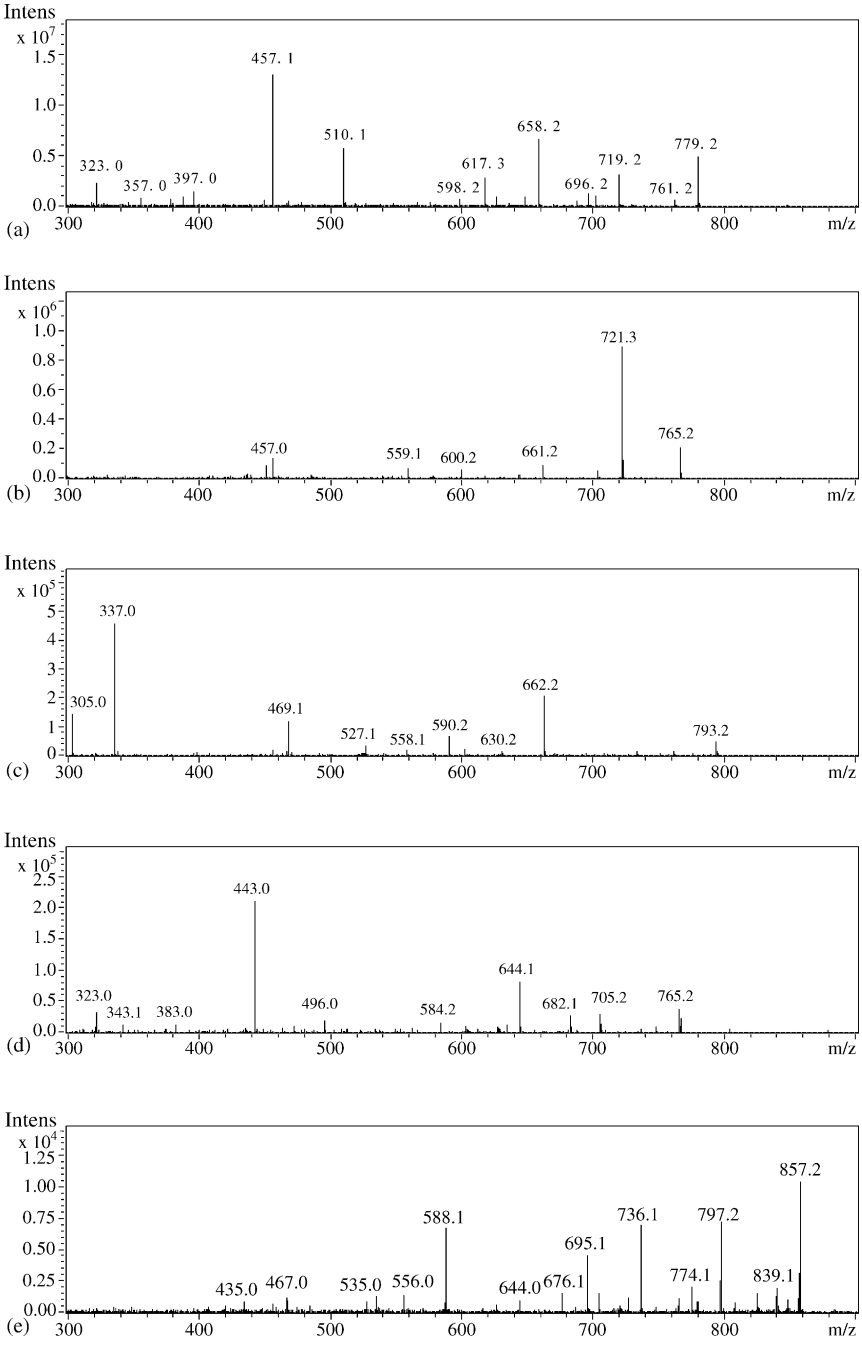

Fig. 4. Positive ion mode HPLC/ESI-MS/MS analyses of vinorelbine, impurity-1, impurity-2, impurity-3 and impurity-4, (a) ESI-MS/MS of parent ion at *m*/*z* 779 for vinorelbine; (b) ESI-MS/MS of parent ion at *m*/*z* 765 for impurity-1; (c) ESI-MS/MS of parent ion at *m*/*z* 793 for impurity-2; (d) ESI-MS/MS of parent ion at *m*/*z* 765 for impurity-3; (e) ESI-MS/MS of parent ion at *m*/*z* 857 for impurity-4.

3.2.1. Structure elucidation of impurity-1

The positive HPLC/ESI-MS spectrum (Fig. 3b) of impurity-1 showed an obvious ion signal at *m*/*z* 765, which is 14 mass units less than protonated molecular ion of vinorelbine. According to the mass spectrometric shift technique [13], this could be attributed to the methyl elimination from vinorelbine. The positive HPLC/ESI-MS/MS spectrum (Fig. 4b) for parent ion at *m*/*z* 765 of impurity-1 showed characteristic fragment ions at *m*/*z* 721 $[M + H{-}CO_2]^+$, 600 $[M + H{-}CO_2{-}C_8H_{11}N]^+$, which implying the presence of carboxyl in impurity-1. On the other hand, comparing to vinorelbine, the positive ESI-MS/MS scan generated a common fragment ion with *m*/*z* 457 corresponding to the vindoline fragment. In addition, the high-resolution mass spectrum of impurity-1 showed the protonated molecular ion $[M + 1]^+$ at *m*/*z* 765.3853, which is compared to the calculated value at

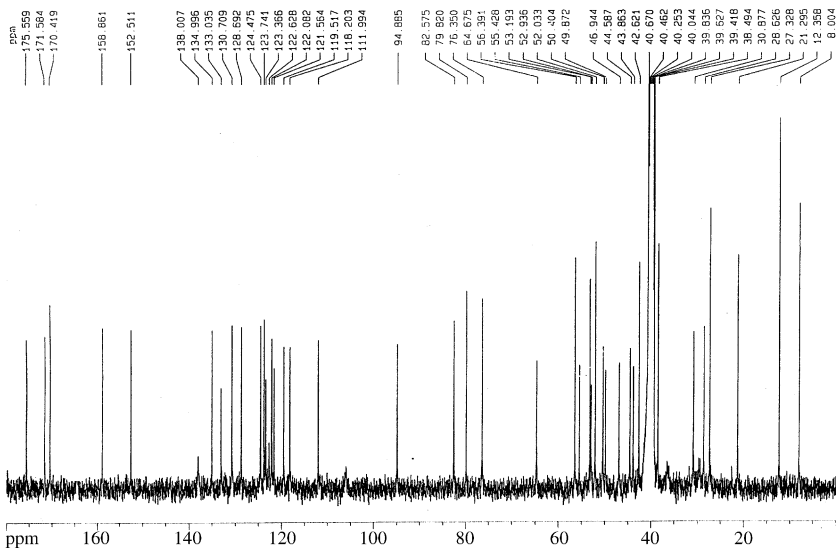

Fig. 5. ^{13}C NMR spectrum for impurity-1 in d_6-DMSO.

765.3858, implying the elemental composition of protonated impurity-1 is $C_{44}H_{53}O_8N_4^+$. Thus, impurity-1 was identified as 18′-*O*-demethylvinorelbine.

The molecular structure of impurity-1 was confirmed by its 1D and 2D NMR spectra. The ^{1}H NMR and ^{13}C NMR (Fig. 5) spectral data of impurity-1 are very similar to that of vinorelbine, except for the proton signal at 3.68 ppm (3H, OCH_3) and the corresponding carbon signal at 53.2 ppm (OCH_3) are absent. It was assumed that the impurity-1 consists of the same molecular framework as vinorelbine, with a hydrogen atom in place of the methyl of 18′-$COOCH_3$ in catharanthine portion. The absence of coupling between the ester methyl proton resonance at 3.68 ppm and a carbon at 175.6 ppm in HMBC spectrum of impurity-1 further lends support to the methyl elimination from 18′-$COOCH_3$.

The presence of impurity-1 in the bulk drug of vinorelbine bitartrate sample was rationalized maybe resulting from ester hydrolysis in synthetic process of vinorelbine bitartrate [1]. As known to us, the impurity-1 is first reported as the process impurity for vinorelbine bitartrate.

3.2.2. Structure elucidation of impurity-2

The positive HPLC/ESI-MS spectrum (Fig. 3c) of the impurity-2 displayed an obvious ion signal at *m*/*z* 793, which is 14 mass units more than vinorelbine. There is a key fragment ion at *m*/*z* 337 in MS/MS spectrum (Fig. 4c), which is the same as that of the naturally occurring catharanthine. In combination with 1D NMR data, the impurity-2 can be explicitly confirmed as 6′-*N*-methylvinorelbine.

There is an additional singlet signal at 3.02 ppm (3H) in ^{1}H NMR spectrum comparing to vinorelbine. In the ^{13}C NMR spectrum, the corresponding carbon signal is present at 51.6 ppm, which is assigned as a methyl carbon according to DEPT (135) spectrum. Moreover, the obvious chemical shifts changes of the groups connected to 6′-*N* in catharanthine moiety are observed. Their chemical shifts at 52.2, 46.7 and 43.5 ppm in vinorelbine are shifted to 61.7, 54.8 and 53.1 ppm for impurity-2, respectively. All these data further support to the *N*-methyl addition on 6′-*N* within catharanthine moiety.

3.2.3. Structure elucidation of impurity-3

The potential molecular ion $[M+H]^+$ of impurity-3 was recorded at *m*/*z* 765 (Fig. 3d), which was the same as the case of impurity-1, implying a hydrogen atom replaces a methyl. The positive ESI-MS/MS spectrum for parent ion at *m*/*z* 765 of impurity-3 was shown in Fig. 4d. As shown in Fig. 4d, there are key fragment ions at *m*/*z* 705 $[M+H{-}HCOOCH_3]^+$, 644 $[M+H{-}C_8H_{11}N]^+$ and 443 (modified vindoline fragment), which are 14 mass units shift from those of vinorelbine, and 323 (catharanthine fragment). These fragment patterns indicated the impurity-3 encompass the same molecular skeleton with losing a methyl on vindoline moiety.

The site of impurity-3 modification within the vindoline moiety could be further localized by its 1D NMR spectrum. Comparing to 1D NMR data of vinorelbine, a singlet of methyl at 3.64 ppm (3H) and the corresponding carbon signal at 52.1 ppm are absent in ^{1}H NMR and ^{13}C NMR spectrum of the impurity-3, respectively, which lends support to the methyl loss from 23-$COOCH_3$ in vindoline portion.

According to the above data, the structure of impurity-3 was confirmed as 23-*O*-demethylvinorelbine.

3.2.4. Structure elucidation of impurity-4

At the point of the structure elucidation of impurity-4, the positive ESI-MS spectrum (Fig. 3e) played an important role as it provided the ions peaks at *m*/*z* 857 and 859 with approximate 1:1 ratio, which are 78 and 80 mass units higher than vinorelbine protonated molecular ion, respectively. The above phenomenon is likely to be attributed to a bromine atom substitution on vinorelbine molecule. The product ion spectrum of precursor ion at *m*/*z* 857 was displayed in Fig. 4e.

As can be seen, the impurity-4 yielded key fragment ions at *m*/*z* 797 $[M + H{-}HCOOCH_3]^+$, 736 $[M + H{-}C_8H_{11}N]^+$, 535 (modified vindoline fragment), 78 mass units shift comparing to those of vinorelbine, which revealed important details on structural similarity of impurity-4 and vinorelbine. Also, it indicated a bromine atom substitution on vindoline moiety. Scanning on the precursor ion *m*/*z* 859, the product ion spectrum showed similar fragment ions with 2 mass units higher than those of the precursor ion *m*/*z* 857, which confirmed a bromine atom substitution on vindoline moiety.

Additional evidence for identity of impurity-4 was provided by its 1D NMR spectra. In its ^{1}H NMR spectrum, an aromatic proton signal at 6.40 ppm (1H) was absent comparing to that of vinorelbine. Further, in ^{13}C NMR spectrum, the quaternary carbon signal at 100.8 ppm displaced methine carbon signal at 94.3 ppm in comparison with vinorelbine. Taking into consideration the similarities and differences between impurity-4 and vinorelbine, we concluded that impurity-4 possessed a vinorelbine-like structure with a bromine atom substitution on C-17 atom of vindoline moiety. Based on the above data the structure of impurity-4 was identified as 17-bromovinorelbine.

4. Conclusions

A simple and sensitive high-performance liquid chromatograph coupled with mass spectrometry method has been set up to separate and identify impurities in semi-synthetic vinorelbine bitartrate sample, which is useful to control the quality of bulk drug of vinorelbine bitartrate. Despite the complexity of the structures for vinorelbine group, applying mass spectrometric shift technique to ESI-MS/MS analysis, structures of four impurities have been unambiguously identified. Moreover, the impurity-1 identified as 18′-*O*-demethylvinorelbine is first reported as a process impurity for vinorelbine bitartrate.

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