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TLC and HPLC Methods to Follow the Synthesis of Vinorelbine

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Abstract

The thin-layer chromatography (TLC) combined with high-performance liquid chromatography (HPLC) has been proved to be a quick and valid method to detect the intermediates and the end-product created during the chemosynthesis process of vinorelbine (VB). This paper gives a detailed investigation on the results of two determination methods when the condition of the detection changed. It shows that when TLC developer is consisted of petroleum ether, chloroform, acetone, and diethyl amine (23.5:12:2:2.5, v/v/v/v), vinblastine sulfate (VBS), anhydrovinblastine (AHVB), and VB can be separated specifically. When the mobile phase of HPLC is a mixture of methyl alcohol, acetonitrile, diethyl amine, and high purity water (420:252:3:225; v/v/v/v), adjusted with orthophosphoric acid to pH 6.5, the intermediates and the resultants of the chemosynthesis of VB can be determined effectively. It can also be used to fix quantify of the resultants. The calibration curve for VB shows good linearity in the two mass concentration ranges of 0.0100–0.0500 mg/mL (r = 0.9956) and 0.00600–0.0100 mg/mL (r = 0.9978), respectively. The limit of detection of HPLC for VB is 0.200 µg/mL.

Introduction

Vinorelbine (VB, Figure 1) is a novel semisynthesis vinca alkaloid that shows a special cytoinhibitory activity to various kinds of cell tumors because of its antikaryokinesis characteristic. Vinblastine (VBS, Figure 1) and vincristine (VC), isolated from the plant *Catharanthus roseus*, were also proved therapeutically to be effective in the treatment of neoplastic diseases (1). But in comparison with these vinca alkaloids, VB shows that it has a broader anti-spectrum and less neurotoxicity (2). Therefore, as a much more secure antitumor medicine, VB has been widely used in clinical study.

VB shares a similar dimeric structure composed of catharanthine and vindoline with VBS and VC. VB has been so far obtained mainly from catharanthine and vindoline or directly from vinblastine sulfate of natural extracts by further multistep chemical modification (Figure 2). Considering from economical and easily obtaining points, the starting materials of VBS, which was used for the synthesis of VB was with a purity of less than 65%. Moreover, the diversity of transformation from VBS to VB often created various derivatives of VBS. Anhydrovinblastine (AHVB) and *N*-bromosuccinimide appear only as the key intermediates, depending on the conditions and control of the reaction processes. These ensure the tracking and production quality of the desired compounds in such a complex reaction mixture that is both delicate and crucial. In order to provide a better

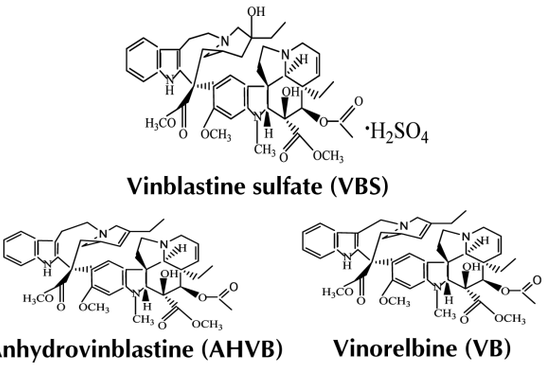

Figure 1. Formulation of the intermediates and end-product of chemosynthesis of VB.

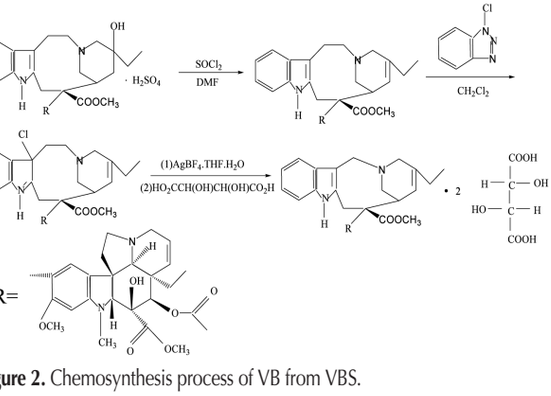

Figure 2. Chemosynthesis process of VB from VBS.

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response to the synthesis and preparation of the desired compounds, the analytical methods are required to be fast, accurate, and without complex preparation of sample as well. However, a quick and effective determination method of VBS, VB, and those key intermediates is rarely available.

Thin-layer chromatography (TLC) is an inexpensive, easy, and sensitive separation method used in various fields, especially in the case of the samples needed to be quickly analyzed and might damage or destroy the columns of LC (3–5). Using reversed-phase high-performance liquid chromatography (HPLC) to separate and detect VB has advantages of high sensitivity and precision. Mouchard et al. (6) chose phosphate buffer–acetonitrile–methanol (50:30:20, v/v/v) (pH 3) as mobile phase, and the limit of detection was 1.00 ng/mL. Other constitutions of mobile phase that have been reported are acetonitrile–80.0 mM ammonium acetate (50:50, v/v) adjusted to pH 2.5 with hydrochloric acid (7), acetonitrile and 0.0500 mol/L potassium dihydrogen adjusted to pH 4.0 with phosphoric acid (8), or water–diethylamine (986:14, v/v) adjusted to pH 7.2 with phosphoric acid as solvent A and methanol–acetonitrile (4:1, v/v) as solvent B. The mobile phase was composed of 380 mL A and 620 mL B (9), methanol–acetonitrile–0.0250 mol/L ammonium acetate–triethylamine. (15:40:45:0.1, v/v/v/v) (10), or methanol 0.00500 mol/L ammonium dihydrogen phosphate adjusted to pH 7.0 (70:30, v/v) (11). The quantitative analysis of VB and VBS were mainly injected into plasma, blood, or in the plant extracts samples. Few works was reported on the determination methods of series products that created during the chemosynthesis process of VB.

In this paper, we describes a combined method of TLC and HPLC, which makes use of the quick and easy-to-operate advantage of TLC and HPLC with more decisive quantification to track the reagents, main products, and the key intermediates. Subsequently, the combined method cannot only give both qualitative and quantitative analysis to the objects but also give gist to the separation and purification followed by the chemosynthesis.

Experimental

Chemicals and reagents

VBS (65%) was supplied by Hai Kou Hai Ning Refined Chemical, and the vinorelbine tartrate was supplied by J & K Chemical (Beijing, China); AHVB and VB samples were obtained from the chemosynthesis. Petroleum ether, chloroform, acetone, diethyl amine, methylene dichloride, and phosphoric acid were purchased from Shanghai Shi Yi Chemicals Reagent (Shanghai, China). All of the reagents mentioned earlier were analytical grade. The HPLC-grade methyl alcohol was obtained from Fisher (Waltham, MA), and HPLC-grade acetonitrile was purchased from Merck (Hong Kong, China); high-purity water was obtained from Hangzhou Wahaha (Hangzhou, China). GF254 silica gel was supplied by Qingdao Hai Yang Chemical Factory (Qingdao, China). The referent substances of VBS, AHVB, and VB were bought from J&K Chemical.

The pH values of the mobile phase (5.5, 6.0, 6.5, 7.0, and 7.5) was determined by pH testing paper (in the range of 5.5 to 9.0), which was supplied by Shanghai San Ai Si Chemicals Reagent (Shanghai, China).

Instruments

Chromatographic analyses was performed on a Waters HPLC apparatus consisting of a 510 pump and a 486 UV detector (Milford, MA). The integration of chromatograms was realized with a N2000 integrator, which was supplied by Zhejiang University. The column employed was a Diamonsil Hypersil ODS C_{18} (250 × 4.6 mm, 5-µm particle size; Lake Forest, CA). The structure confirmation of compounds VBS, AHVB, and VB was performed in the LC–electrospray ionization (ESI)-mass spectrometry (MS) system platform 1100 LC/MSD Trap (Agilent, Santa Clara, CA).

Reference substance preparation

Stock solution of VBS (1.00 mg/mL) and VB (1.00 mg/mL) for TLC were prepared with methyl alcohol (chromatographically pure). The standard working solution of VBS (0.0200 mg/mL) for HPLC was prepared by diluting of the stock solution with mobile phase. The standard working solution of VB (0.0500 mg/mL) was prepared by dissolving with 1.00 mL mobile phase. Other 11 standard working solutions of VB ranging from 0.500 µg/mL to 0.0400 mg/mL were prepared by appropriate diluting of the 0.0500 mg/mL standard working solution of VB with mobile phase. All the solutions mentioned were stored at 4°C.

The identification of compounds VBS, AHVB, and VB in sample were made by Rf values in TLC, retention time in HPLC, and relative molecular weights given by LC with ESI-MS. Mass detection of the sample was made with ESI in the positive ion mode. The source temperature was 200°C ion energy 0.5 V, and capillary voltage 4.5 kV. The drying gas was N^2 at a flow rate of 40 psi. The Q1 mass of ESI-MS are *m/z* 811.6, 793.7, 779.6, respectively, which correspond to M+1 peaks of compounds of VBS, AHVB, VB .

Sample preparation

Stock solutions of AHVB and the end-products for TLC were prepared extemporaneously with methylene dichloride in a concentration of about 1.00 mg/mL. Stock solutions of AHVB and the end-products for HPLC were prepared extemporaneously in mobile phase in the concentration between 0.0100–0.0200 mg/mL. Both the samples of AHVB and VB were obtained from the chemosynthesis of VB.

TLC analysis

The silica gel plate was prepared firstly dissolving 30.0 g GF254 silica gel with 90.0 mL sodium carboxymethyl cellulose (0.500 g/100 mL) and agitating until it became mash, then spreading it on the plates prepared and drying them up in the open air. The plates should be activated at 105°C for 1 h and stored in the desiccator.

Different developers were prepared in order to find out a suitable one that not only has good separability but also can be used to identify both reactants and resultants. Developers of the TLC consisting of chloroform–methyl alcohol (95:5, v/v); chloroform–methyl alcohol–petroleum ether (9:1:5, v/v/v); ethyl

acetate–absolute ethyl alcohol (3:1, v/v); and petroleum ether–chloroform–acetone–diethyl amine (23.5:12:2:2.5; v/v/v/v) were tested. The solutions of the samples were spotted more than three dips on every TLC plate with a 5-µL microcap. The analysis of each sample for TLC was repeated three times. Repeatability was expressed as relative standard deviation (RSD). After development, the plates were evaluated visually under UV light (254 nm).

Limits of detection (LOD) under the TLC procedures was defined as being the lowest amount below which the spots in the developed plates could not be evaluated during the between-run analysis. For LOD, 1–3 dips of a 5-µL microcap for the referent solution of VBS, AHVB, and VB were spotted and developed.

HPLC analysis

Different constitutions of mobile phase have been evaluated in order to find out a suitable one on the basic of the references (7,8). In the meantime, ammonium acetate took place of the diethyl amine, and the composition of the mobile phase was as follows: methyl alcohol, acetonitrile, diethyl amine, and high purity water (420:252:3:225; v/v/v/v), adjusted with orthophosphoric acid to pH 6.5. The solution A was purity water–diethyl amine (225:3) adjusted to pH 6.5 with orthophosphoric acid and then filtered through 0.45-µm microporous filter membranes. Solution B was methyl alcohol–acetonitrile (420:252). Then solution A and solution B were mixed at the rate of 228:672 and deaerated ultrasonically prior to use. The eluent was pumped through the column at an isocratic flow rate of 1.00 mL/min; the injection volume was 10 µL, and the eluate was monitored at 267 nm.

The effect of pH value of the mobile phase was evaluated by comparing the separation ability of mobile phase with different pH value, which was adjusted by adding orthophosphoric acid or diethyl amine to get from pH 5.5 to 7.5.

Results and Discussion

TLC analysis

The starting material of chemical reaction was composed of 65% VBS. Judging from the effect of TLC, there were no less than three main impurities in the starting material. The spots of three impurities on the TLC silica gel plate may affect the detection result of tracking the chemosynthesis process. Therefore, the separation ability of four main components of starting material sample is one of the evaluation standards in choosing the developer.

Four kinds of developers were experimented to investigate separation capability. In Table I, the results show that when TLC developer consisted of petroleum ether, chloroform, acetone, and diethyl amine, VBS, AHVB, and VB can be best separated. Even though the developer of the composition of chloroform–methyl alcohol (95:5, v/v) can also separate the three objects, experiments showed that the four components of starting material sample can't be separated totally in that case. While keeping the constant concentrations of chloroform and acetone, increasing the concentration of petroleum ether, or decreasing the concentration of the diethyl amine, the Rf of four components of VBS increased. And while the concentration of the petroleum ether arrived at 46% or more, two impurities (impurity B and impurity C) cannot be separated on the silica gel plate (Table II).

When using petroleum ether–chloroform–acetone–-diethyl amine (23.5:12:2:2.5, v/v/v/v) as developer to track the chemosynthesis process, the spot of VBS disappeared while the spots of the other three impurities still existed after the first synthesis step. The spot of AHVB appeared, and two other spots appeared as new spots during the whole synthesis process, TLC showed a good resolving power to object, impurities, and concomitant products, and it can give a quick and believable detection to the process. Therefore, choosing petroleum ether–chloroform–acetone–diethyl amine (23.5:12:2:2.5, v/v/v/v) as developer was proved to be the most suitable one.

The RSD for repeatability of the objects were calculated from Table I and Table II to be below 5%, which indicated a good repeatability. Without quantitation, LOD of the TLC method is still necessary to estimate the lowest amount of spotting that can be detected. In this investigation it was calculated to be 5 µg. Considering simplicity, quickness, and low costs, the TLC method could be conveniently used for detection, for even semi-quantitative estimation of the precursors, intermediates, by-products, and the end-product during the synthesis of VB.

HPLC analysis

Though TLC can give a quick evaluation of the chemosynthesis process, HPLC, which gives a quantitative analysis, plays

Table I. Effect of Different Developers on the Separation of VBS, AHVB, and VB

Constitute of developer (v:v)	Rf of VBS, AHVB, and VB		
	VBS	AHVB	VB
Chloroform–methyl alcohol (95:5)	0.40 ± 0.01	0.33 ± 0.01	0.30 ± 0.01
Chloroform–methyl alcohol–petroleum ether (9:1:5)	0.36 ± 0.01	0.34 ± 0.02	0.34 ± 0.02
Ethyl acetate–absolute ethyl alcohol (3:1)	0.30 ± 0.01	0.33 ± 0.02	0.18 ± 0.01
Petroleum ether–chloroform–acetone–diethyl amine (23.5:12:2:2.5)	0.42 ± 0.01	0.37 ± 0.01	0.31 ± 0.01

Table II. Effects of Different Concentration of the Four Components of the Developer

Conc. of the four components in the developer (v/v/v/v)*	Rf of the four main components of source material sample			
	Impurity A	VBS	Impurity B	Impurity C
24:12:2:2	0.14 ± 0.01	0.25 ± 0.01	0.35 ± 0.01	0.42 ± 0.02
23.5:12:2:2.5	0.30 ± 0.01	0.42 ± 0.02	0.52 ± 0.01	0.59 ± 0.02
23:12:2:3	0.40 ± 0.02	0.56 ± 0.01	–†	–
22:12:2:4	0.50 ± 0.01	0.69 ± 0.02	–	–

* Petroleum ether–chloroform–acetone–diethyl amine (v/v/v/v).
† Cannot be separated.

another important role in the determination.

Different mobile phases were used to detect VBS, AHVB, and VB. Results showed that by increasing the concentration of organic phase, the retention times of VBS, AHVB, and VB would be brought forward. The adding of diethyl amine as a component of mobile phase would help the separation of each peak. Some references reported that using ammonium acetate to take place of diethyl amine as one part of the mobile phase would have a good separation of each component, but we found that the base line was not smooth and the peaks of the main objects have trail formation.

Methyl alcohol–acetonitrile–diethyl amine–high purity water (420:252:3:225, v/v/v/v), adjusted with orthophosphoric acid to pH 6.5, was experimented to be the most suitable mobile phase to analyze the reactants and the products. The observed retention times of VBS, AHVB, and VB sample was 11.5, 32.4, and 24.6 min, respectively (Figure 3).

The chromatograms of VBS, AHVB, and VB, which were created in the chemosynthesis process, were overlained together (Figure 4). It demonstrated that the three components could be separated totally; the relative retention time for each component was 0.356, 1.00, and 0.760, respectively.

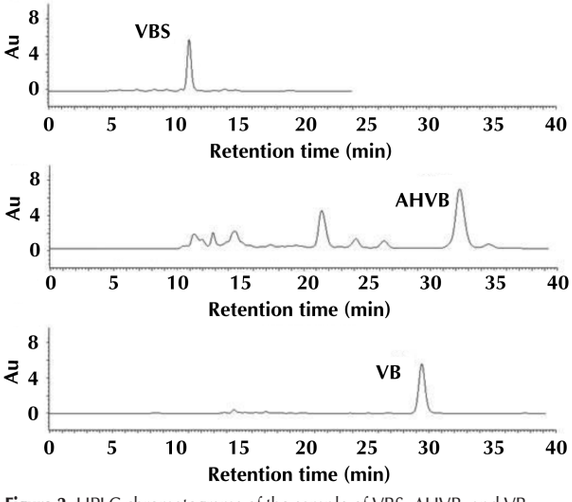

Figure 3. HPLC chromatograms of the sample of VBS, AHVB, and VB.

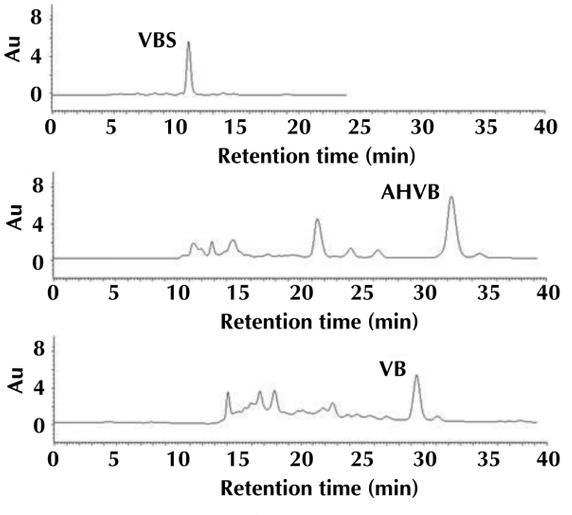

Figure 4. HPLC chromatograms of VBS, AHVB, and VB that are created in the chemosynthesis process.

During the course of detection of intermediates and the end-products of the chemosynthesis of VB, VB was compared with the reference substance and proved to be the object that we needed. For AHVB, it was identified by LC–MS.

pH of the mobile phase varies from 5.5 to 7.5 have also been tested to detect their separation ability on VBS, AHVB, and VB. Figure 5 shows that the retention factors (k') of object changed as pH of the mobile phase altered. At the condition of acidity or neutrality, when pH of the mobile phase increased, the retention time of the objects delayed. It is obvious especially for VB. When pH was 5.5 or 6.0, the baseline of chromatogram of VBS and AHVB was not smooth, and some peaks could not be baseline separated. However, while the pH of the mobile phase arrived at 7.0 or higher, it took a long time to detect VB and AHVB. It's a waste of the mobile phase and would do harm to the column because of high alkalescence. Therefore, adjusting pH of mobile phase to 6.5 would be the best choice.

Other paper reported using this composition of mobile phase (pH 7.2) can separate vinblastine, vincristine, catharanthine, and vindoline successfully, but whether this mobile phase was suitable to analyze the intermediates and the end-products of the chemosynthesis of VB was not studied. While using the mobile phase according to the report, we found that it took a long time to detect the objects that created in the synthesis. Adjusting the pH value of the mobile phase and the concentration of each component would be much better for the detection of the synthesis.

From the molecular structural, compound AHVB contains a extra methylene group at positions 3',4' compared with VB and VBS. As a result, AHVB has the biggest reversed-phase retention values among the three compounds (Figure 3). Moreover, VBS has a hydroxyl near the 3' alkyl group compared with AHVB and VB so that it appears earlier in Figure 3 than the other objects. The retention order of these compounds by TLC of Rf data would be AHVB > VB > VBS when using normal phase. But the order in the Table I indicated AHVB > VBS > VB when using petroleum ether–chloroform–acetone–diethyl amine (23.5:12:2:2.5, v/v/v/v) as developer. This was not in accordance with the previous explanation based on structure. But the TLC retention order of VB and VBS was consistent with the results of reported by Paci et al. (12), who identified and quantified the four vinca-alkaloids including VB and VBS using HPTLC.

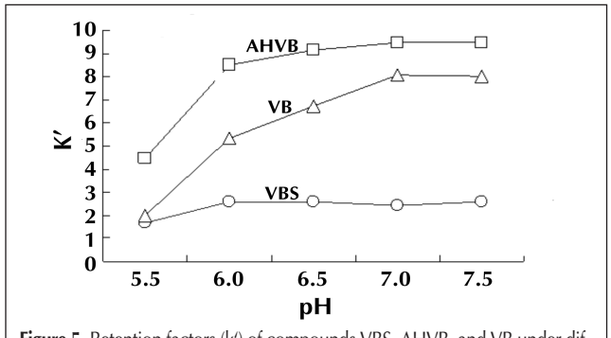

Figure 5. Retention factors (k') of compounds VBS, AHVB, and VB under different pH mobile phase.

Because HPLC determination was sensitive to pH value of the mobile phase, it gave an elicitation to separation of the products followed by chemosynthesis. Because adding diethyl amine would help the separation of the impurities and the objects, controlling the pH of the eluent and adding proper amine salt could be considered on the separation of the components of the intermediates and end-products. Because reversed-phase HPLC has the advantages of high selectivity and sensitivity, we could choose reversed-filling agent to separate and prepare for high purity VB after the chemosynthesis.

The linearity of the HPLC method was evaluated by analyzing working solutions of VB. Eleven solutions diluted to various concentrations (0.500, 1.00, 2.00, 4.00 , 6.00, 8.00, 10.0, 20.0, 30.0, 40.0, and 50.0 µg/mL) were prepared in order to determine the relationship between the peak area and the concentration of analyte. Results indicated that the peak area of VB over the internal standard varied linearly with concentration over the range used. They were linear in the investigated ranges and described by the following equations: $y = 2 \times 107x - 14535$ ($r = 0.9956$) over five concentration points; $y = 107x + 278.59$ ($r = 0.9978$) over three concentration points for the ranges 10.0–50.0 µg/mL and 6.00–10.0 µg/mL, respectively, where y is peak-area and x is concentration (mg/mL). When concentration of the standard solutions fall below 0.500 µg/mL, the linearity of the HPLC method was not satisfied.

Accuracy was expressed as the recovery, while the precision was given by the inter-day RSD. Standard sample was added into four different concentration samples (0.0500, 0.0200, 0.0100, 0.00800 mg/mL), and the corresponding recovery was 99.4%, 102.3%, 100.2%, and 99.7%, respectively.

Precision of the method was evaluated by performing replicate analysis of VB samples at four concentrations (0.0500, 0.0200, 0.0100, 0.00800 mg/mL). Each sample was analyzed six replicates on the same day to determine intra-day precision. The RSD of the peak area of four concentrations are 1.43%, 1.30%, 1.46%, and 0.70%, respectively. All of them are below 2%.

The LOD of HPLC method was defined as the sample concentration resulting in a peak area of three-times the noise level. The LOD was 0.200 µg/mL.

Conclusion

A determination method that combined HPLC and TLC together was optimized and evaluated for identification of the intermediates and the end-product created during the chemosynthesis of VB.

When TLC developer was constituted of petroleum ether–chloroform–acetone–diethyl amine (23.5:12:2:2.5; v/v/v/v), and the mobile phase of the HPLC was a mixture of methyl alcohol, acetonitrile, diethyl amine, and high purity water (420:252:3:225; v/v/v/v), adjusted with orthophosphoric acid to pH 6.5, VBS, AHVB, and VB could be separated and detected easily.

Results demonstrated that using TLC to track the chemosynthesis process of VB is much quicker and more convenient. When this method was combined and reinforced by HPLC, the new combined method can make qualitative and quantitative analysis to the chemosynthesis of VB more effectively because of its advantage of quickness, precision, and convenience.

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