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Short communication

Sensitive high-performance liquid chromatographic method with fluorescence detection for measurement of vinorelbine plasma concentrations

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Abstract

A high-performance liquid chromatographic (HPLC) method with fluorescence detection for the determination of vinorelbine in plasma is described. The technique was derived from that published by Debal for an assay of vinorelbine in cell culture medium. The modifications concern the preparation procedure for plasma samples (a two-step liquid–liquid extraction from plasma is described), optimization of the mobile phase composition, and use of a single C_{18} column. These changes resulted in an improved sensitivity and reproducibility of the assay and led to its feasibility for clinical pharmacokinetic studies. The range of the assay is 2 to 1000 ng/ml.

Keywords: Vinorelbine

1. Introduction

Vinorelbine (VRB) is a new semi-synthetic vinca alkaloid (Fig. 1). This drug shows reduced neurotoxicity compared with other vinca alkaloids. Its antitumor activity has been demonstrated in a variety of malignancies (metastatic breast cancer, lung cancers, lymphomas, etc.). Its relative safety and the large spectrum of its antitumor activity explain why the clinical use of VRB is rapidly expanding [1,2]. A very sensitive detection method is required for pharmacokinetic studies as its plasma concentrations are

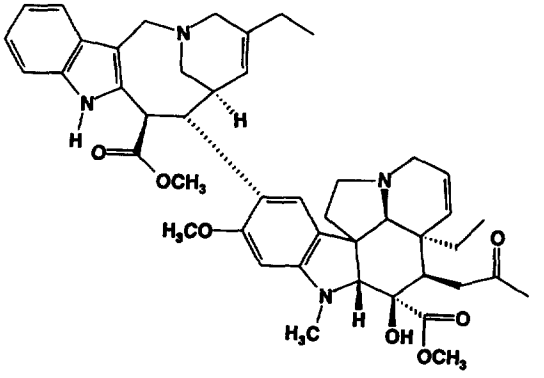

Fig 1. Chemical structure of vinorelbine.

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typically below 5 ng/ml 24 h after a standard dose of 30 mg/m^2. Initially, a radioimmunoassay was used [3]. Cross-reactivity with metabolites resulted in an over-estimation of the VRB concentration, limiting the interest in this method. Several HPLC methods have been published for the assay of VRB in plasma, urine, tumor cells, and cell culture medium [4–7]. We wished to study the pharmacokinetics of VRB in groups of patients at high risk for toxicity. We faced difficulties in reproducing published methods for assay in plasma [4–6]. In 1990, Jehl et al. [4] developed an HPLC method with UV detection. A simple extraction method using diethyl ether was described. With this method we obtained a very high solvent front and noisy baseline, resulting in poor precision and reproducibility at concentrations below 10 ng/ml. Our investigations suggested that these difficulties were due to interferences with plasma components, and that a more selective detection and/or better clean-up procedure were needed. The same year Nicot et al. [5] published a very sensitive method based on electrochemical detection. However, we preferred to use fluorescence detection which is equally sensitive but more robust. In 1992 Van Tellingen et al. [6] published an HPLC assay with fluorescence detection, but the sensitivity of the assay (5 ng/ml) was deemed insufficient for our purposes. We therefore elected to adapt the method developed by Debal et al. [7] for cell culture medium in order to improve its sensitivity and obtain a reliable dosage of plasma concentrations for clinical pharmacokinetic applications.

2. Experimental

2.1. Chemicals and reagents

Vinorelbine (VRB) and vinblastine (VLB) were supplied by Farmitalia (Milan, Italy). Both drugs were stored as a methanol solution (stock solution 0.5 mg/ml; work solution 0.05 mg/ml). In the freezer (−20°C) these solutions were stable for at least six months. Non-stabilized diethyl ether for fluorimetry, methanol (HPLC grade), sodium dodecyl sulphate, and potassium phosphate were purchased from Carlo Erba (Milan, Italy), HPLC-grade acetonitrile from J.T. Baker (Deventer, Netherlands), and phosphoric acid from Merck (Darmstadt, Germany). Water was deionized by the Milli-Q Water System from Millipore (Vimodrome, Italy).

2.2. Chromatographic conditions

The chromatographic system was from Waters and consisted of a Model 510 pump, an autosampler 717 plus, equipped with a 200- μl loop and a scanning fluorescence detector Waters 470. The excitation wavelength was set at 280 nm and emission at 360 nm. Data were acquired, stored, and processed with the chromatography data management software Millennium 2000 (Waters). Separation was performed with a single silica analytical column (Novapack C_{18} Waters; 300 × 3.9 mm I.D.). To protect the analytical column, we used a pre-column (Waters, Novapack C_{18}) fixed in-line with a guard-pack holder. The mobile phase was eluted at 0.9 ml/min with a back pressure of about 21 MPa. The mobile phase was a mixture of acetonitrile and phosphate buffer (60:40, v/v). The buffer was prepared with 75 m*M* phosphoric acid and monobasicpotassium phosphate (5:1, v/v) containing 0.1 g/l of sodium dodecyl sulphate and adjusted to pH 2.70 using 2.5 *M* phosphoric acid.

2.3. Extraction from plasma samples

The extraction of VRB from 2 ml of plasma was performed in two liquid–liquid extraction steps. After addition of the internal standard (20 μl of a 0.5 μg/ml solution of vinblastine), a first extraction was performed with 8 ml of diethyl ether in glass, PTFE screw-cap tubes. After slow agitation for 45 min, centrifugation at 2000 *g* for 5 min and congelation, the supernatant organic phase was decanted into polyethylene tubes and evaporated to reduce the ether volume to 1 ml. A second, acidic extraction was then performed, using 220 μl of 75 m*M* phosphate buffer at pH 2.7. After slow agitation for 45 min the acidic aqueous extract (200 μl) was injected into the HPLC system.

2.4. Calibration curves

Quantitation was based on the internal standard method, using the ratio of peak areas and two calibration curves. For low concentrations, 2 ml of plasma were spiked with 10 ng of VBL and 2.5, 5, 10, 25 ng of VRB. For high concentrations, 1 ml of plasma was spiked with 10 ng of VBL and 25, 50, 100 and 500 ng of VRB. Extracted calibration curves and two quality-control samples were run every day for each set of twenty-eight unknown plasma samples (two patients).

2.5. Clinical pharmacokinetics application

We studied the pharmacokinetics of vinorelbine in five patients (mean age 56.7 ± 8 years) with advanced breast cancer treated by weekly intravenous vinorelbine at standard doses (30 mg/m^2) administered by a 20-min infusion. Fourteen heparinized blood samples were drawn during a four-day hospitalization period (0.5, 0.75, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96 h after the beginning of infusion). After the blood was centrifuged, the plasma was stored at −20°C until analysed.

3. Results

3.1. Chromatograms

Fig. 2 illustrates the chromatograms obtained (A) from a 2-ml blank plasma sample, (B) from a blank plasma spiked with 12.5 ng/ml VRB and 5 ng/ml VLB, and (C) from a plasma sample obtained 4 h after VRB infusion in a patient. The retention times of VLB and VRB are about 8 and 13 min, respectively, with a good resolution of the two compounds. VRB peak has a lower voltage than the peak corresponding to the same amount of VLB. Resolution from the solvent front was good. The peak eluting at 9 min (Fig. 2C) found in the plasma of some patients treated with VRB and not found in blank plasma is probably a metabolite of VRB.

3.2. Linearity of calibration curves

Linearity was demonstrated for the two calibration curves for low and high VRB concentrations. Excellent correlation coefficients were obtained for aqueous solutions of standards ($r > 0.999$) and from blank plasma spiked with standards ($r = 0.999$). The use of two calibration curves improved the accuracy of the quantitation of low concentrations.

3.3. Recovery

Comparison between calibration curves after extraction from plasma and curves generated from aqueous dilutions of standards was used to calculate the percentage of recovery (87% for VLB and 70% for VRB).

3.4. Limit of detection and quantitation

At the concentration of 1 ng/ml, the VRB peak was consistently detectable, being more than three times above the baseline noise. Limit of quantitation was 2 ng/ml (intra-assay coefficient of variation: 6.05%). Inter- and intra-day variabilities of the assay are reported in Table 1.

3.5. Clinical application

Plasma concentration versus time curve in patient 1 is illustrated in Fig. 3. Peak plasma concentration was observed at 0.5 h (mean

Table 1
Inter- and intra-day variability of the assay

Spiked concentration (ng/ml)	Intra-day coefficient of variation (%)	Inter-day coefficient of variation (%)
1	24.86	
2	6.05	
3	3.88	
5	5.42	16.7
20	1.27	
50	4.24	14.0
100	5.30	
500	5.96	14.2

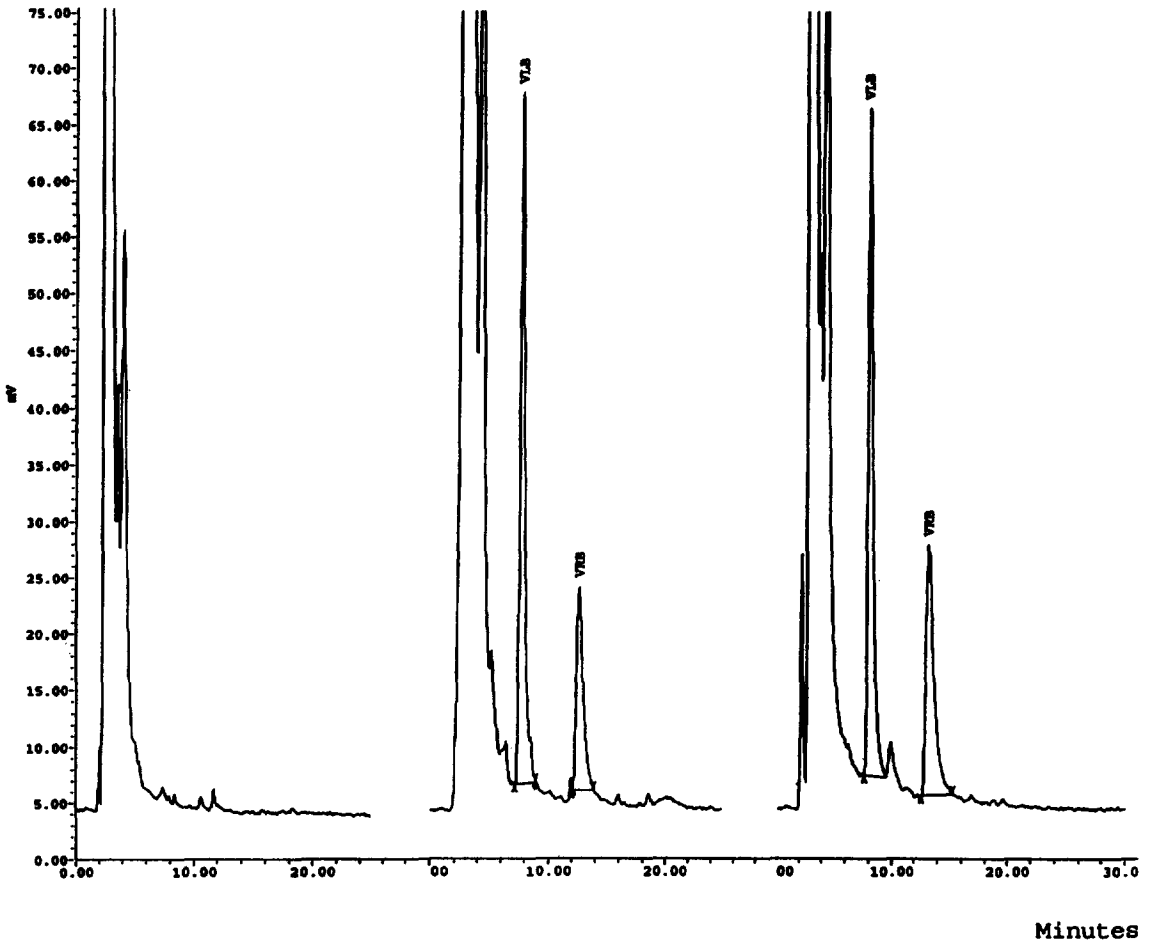

Fig 2. Chromatograms of (A) blank plasma sample, (B) blank plasma spiked with 12.5 ng/ml of VRB and 5 ng/ml of VLB and (C) plasma sample obtained in a patient 4 h after VRB injection (VRB concentration = 23 ng/ml).

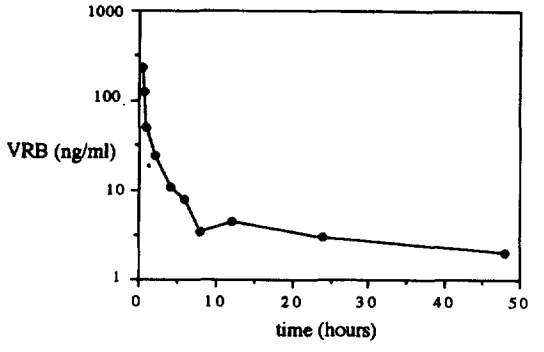

Fig 3. Vinorelbine plasma concentration versus time curve in patient 1.

C_{max} = 179 ± 27 ng/ml). Vinorelbine was above 2 ng/ml for 48 h in four patients and 72 h in the remaining patient. Mean terminal half-life was 25.6 ± 3.5 h.

4. Discussion

In this work, we developed and validated a sensitive and reproducible HPLC assay for measuring vinorelbine in plasma and demonstrated its feasibility for clinical pharmacokinetics study.

4.1. Sample preparation

The extraction method is based on the chemical properties of vinorelbine and vinblastine. Plasma pH (7.2 to 7.4) lies well above the pK_a (5.4) of the weak alkaline function of vinorelbine and vinblastine. In plasma the compounds are predominantly non-ionized and therefore soluble in the organic phase (diethyl ether). Reducing the volume of ether by partial evaporation concentrates the extract and facilitates contact between the organic and aqueous phases in the following extraction step. Evaporation to dryness resulted in a disturbed baseline and an erratic recovery. The second step is an acidic extraction; after addition of the acidic phosphate buffer, the compounds become protonated and concentrated in the aqueous phase that will be injected into the chromatograph. The two-step liquid–liquid extraction from plasma resulted in a more complete extraction of the internal standard (VLB) than of vinorelbine. However, the ratios of recoveries were stable, resulting in accurate quantitation of vinorelbine in the range of the assay.

4.2. Amendments to the original chromatographic conditions

The mobile phase composition in the original method (25 m*M* phosphate buffer adjusted to pH 2.7 with phosphoric acid) needed optimization. We established that a 75 m*M*, pH 2.70 phosphate buffer improved elution time and peak shape. A reduction in the length of the analytical column (300 instead of 450 mm) was necessary to keep the working pressure below 28 MPa, which is the maximum-tolerated pressure for the autosampler. This could be done without altering the resolution or sensitivity. Moreover, the use of a single column is simpler and cheaper. A C_{18} pre-column was used to protect the analytical column; the pre-column was replaced when a rise in operating pressure was observed.

The range of the assay was sufficient for the quantitation of vinorelbine from peak values to concentrations at 48 to 72 h, that is two to three half-lives.

In conclusion, we describe a simple, sensitive and reproducible HPLC method with fluorescence detection for the dosage of vinorelbine in human plasma. A first application to a clinical pharmacokinetics study confirmed the usefulness of this method.

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