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High-performance liquid chromatographic determination of vinorelbine in human plasma and blood: application to a pharmacokinetic study

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Abstract

A sensitive and specific high-performance liquid chromatographic method with fluorescence detection (excitation wavelength: 280 nm; emission wavelength: 360 nm) was developed and validated for the determination of vinorelbine in plasma and blood samples. The sample pretreatment procedure involved two liquid–liquid extraction steps. Vinblastine served as the internal standard. The system uses a Spherisorb cyano analytical column (250×4.6 mm I.D.) packed with 5 μm diameter particles as the stationary phase and a mobile phase of acetonitrile–80 m*M* ammonium acetate (50:50, v/v) adjusted to pH 2.5 with hydrochloric acid. The assay showed linearity from 1 to 100 ng/ml in plasma and from 2.5 to 100 ng/ml in blood. The limits of quantitation were 1 ng/ml and 2.5 ng/ml, respectively. Precision expressed as RSD was in the range 3.9 to 20% (limit of quantitation). Accuracy ranged from 92 to 120%. Extraction recoveries from plasma and blood averaged 101 and 75%, respectively. This method was used to follow the time course of the concentration of vinorelbine in human plasma and blood samples after a 10-min infusion period of 20 mg/m^2 of this drug in patients with metastatic cancer.

Keywords: Vinorelbine

1. Introduction

Vinorelbine, nor-5′-anhydrovinblastine (Navelbine®) is a relatively new semi-synthetic anti-cancer agent of the vinca-alkaloid group showing reduced neurotoxicity compared with other vinca alkaloids. This drug is a dimeric molecule formed by the catharanthine unit indolic moiety and the vindoline unit (dihydroindolic moiety) joined by a carbon–carbon bridge. It is a very lipophilic molecule; hence, the partition coefficient octanol–buffer, pH 7.2 which is related to lipophilicity is 16 [1]. Vinorelbine has anti-tumor activity against a wide spectrum of human cell lines in vitro and in vivo. In patients, a weekly intravenous administration of vinorelbine at a dose of 30 mg/m^2 has shown a good activity in non-operable non-small-cell lung cancer (NSCLC)

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and in advanced breast and ovarian cancers [2–7]. Little is known about the metabolism of vinorelbine. Some studies suggest the existence of three minor metabolites including desacetylvinorelbine. This metabolite represents only 0.25% of the administered dose in urine and has not been detected in human serum [8]. The pharmacokinetic properties of this drug are currently under investigation.

The first pharmacokinetic data of vinorelbine were obtained in man and in animals using radioactive assays [7,10] or radioimmunoassay [9,10]. To date, some high-performance liquid chromatography (HPLC) methods for determining vinorelbine in biological fluids (plasma, urine, tumor cells and cell culture medium) have been published [10–17]. They involved ultraviolet [11,14], fluorescence [13,15,17], scintigraphic [10] or electrochemical [12,16] detection. According to the methods, the extraction of vinorelbine from the matrix was performed in one or two liquid–liquid extraction steps. However, several methods reported assay validation which was incomplete; moreover, none of these methods reported stability assays in biological fluids.

As previously published, the distribution of vinorelbine, in vitro, showed that 84% is bound to blood cells, mainly to platelets (78%) [18]. So, it appears very interesting to be able to determine vinorelbine in blood and to compare the pharmacokinetic properties in plasma and in blood. To date, no method has been validated to quantify vinorelbine in blood.

The present paper describes an isocratic reversed-phase HPLC method to quantify vinorelbine in plasma and blood. Vinblastine was used as internal standard. This method was validated with respect to accuracy, precision, selectivity and limits of quantitation (LOQ) and detection (LOD) according to Good Laboratory Practice Guidelines [19–21]. Moreover, stability tests under various conditions have been performed. This method was used to determine vinorelbine in plasma and blood samples in elderly patients with advanced malignant cancer.

2. Experimental

2.1. Materials and reagents

Vinorelbine bitartrate and vinblastine sulfate (internal standard) were of analytical purity and kindly provided by Pierre Fabre Oncology (Boulogne, France) and by Lilly Laboratories (Saint-Cloud, France), respectively. Acetonitrile, diethyl ether, hydrochloric acid and ammonium acetate were purchased from Merck (Darmstadt, Germany). All of them were liquid chromatography grade. A phosphate buffer solution (pH 7.4, 1 *M*) was obtained from Sigma (Saint Quentin-Fallavier, France) and was 10-fold diluted and adjusted to pH 2.5; it was used for the extraction. For the mobile phase a solution of ammonium acetate (80 m*M*) in sterile water (Fresenius France Pharma, Sèvres, France) was used.

Stock solutions of vinorelbine (1 mg/ml) and vinblastine (1 mg/ml) were prepared in sterile water and stored at −20°C. Working solutions at 10 and 100 ng/ml for vinorelbine, and at 0.5 μg/ml for vinblastine were prepared extemporaneously in sterile water; they were used to prepare calibration curves and quality control (QC) samples. The volume added was always smaller than or equal to 2% of total volume of the samples, so that the integrity of the plasma and the blood was maintained.

An unextracted working solution containing vinorelbine and vinblastine at a concentration of 50 ng/ml in phosphate buffer at pH 2.5 was prepared daily to check the resolution of the chromatographic system.

For the validation of the method, blood from healthy volunteers was collected on sodium heparinate and plasma was obtained by centrifugation at 1000 *g* for 10 min. Blood and plasma were obtained from the Etablissement de Transfusion Sanguine du Languedoc Roussillon (Montpellier, France). The blood was stored at 4°C and plasma at −20°C before use.

2.2. Equipment

The chromatographic system consisted of a Shimadzu Model LC-10AT pump (Kyoto, Japan), a Shimadzu Model RF-10Axl fluorescence detector, a Rheodyne loading valve (Model 7725i) fitted with a 50-μl sample loop (Touzart & Matignon, Paris, France) and a Shimadzu integrator Model C-R5A (chart speed, 10 mm/min). The excitation wavelength was 280 nm and the emission wavelength was 360 nm. HPLC separation was performed on a Spherisorb cyano analytical column (250×4.6 mm

I.D.) packed with 5 μm diameter particles (Waters, Saint Quentin en Yvelines, France).

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile and 80 m*M* ammonium acetate; the aqueous portion was adjusted to pH 2.5 with hydrochloric acid before use. This mobile phase was filtered through a 0.45-μm HV filter (Millipore, Bedford, MA, USA) then deaerated ultrasonically prior to use. In order to optimize the fluorescence and the resolution between vinorelbine and the internal standard, the effect of the acetonitrile concentration in the mobile phase was tested. The capacity factors and resolution between the two drugs were determined with concentrations of acetonitrile ranging from 45 to 55%. The fluorescence intensity from vinorelbine increased with increasing acetonitrile concentration but the resolution between the two peaks decreases. The final choice of acetonitrile concentration, 50% (v/v), was a compromise between response intensity and resolution.

The eluent was pumped through the column at a flow-rate of 1 ml/min, which corresponds to a pressure of about 8.5 MPa (85 bars). The volume injected was 50 μl. Chromatography was performed at ambient temperature (20°C).

2.4. Analytical procedure

2.4.1. Calibration curves and QC samples

Quantitation was based on the internal standard method. Working solutions of vinorelbine were used to spike plasma (1 ml) and blood samples (0.5 ml) in order to obtain calibration standards at concentrations of 1, 2.5, 5, 10, 25, 50, 80 and 100 ng/ml in plasma and at concentrations ranging from 2.5 to 100 ng/ml in blood.

QC samples were prepared at concentrations of 1, 7.5, 20 and 40 ng/ml in plasma and at concentrations of 2.5, 6.5, 20 and 35 ng/ml in blood.

Immediately after preparation, blood samples were stored at −20°C for 12 h in order to hemolyze red blood cells.

2.4.2. Extraction procedure

In a 7-ml screw-capped glass centrifuge tube, to 1 ml of plasma sample (or 0.5 ml of blood diluted with 0.5 ml of sterile water), 20 μl of internal standard (vinblastine, 0.5 μg/ml) and 4 ml of diethyl ether were added and vortex-mixed for 10 s. After speed agitation for 15 min, the tubes were centrifuged at 2000 *g* for 10 min then frozen at −80°C for 10 min. The supernatant organic phase was drawn off and placed in another 7-ml glass tube then diethyl ether was evaporated under a nitrogen stream at 20°C to a final volume of 1 ml. A second extraction was then performed, using 200 μl of phosphate buffer at pH 2.5. After speed agitation for 45 min and centrifugation at 2000 *g* for 10 min, a 50-μl aliquot of the acidic aqueous phase was injected into the column.

2.5. Data analysis

From recorded peak areas, the ratios of the drug to internal standard were calculated. Unweighted least-squares linear regression of the peak area ratio as a function of the theoretical concentrations was applied to each standard curve [formula: $y=b+ax$; where x=concentration (ng/ml), y=peak area ratio, a=slope and b=intercept]. The slopes and the intercepts were compared with zero, and the correlation coefficients with 1. The equation parameters (slope and intercept) of each standard curve were used to obtain concentration values for that day quality control samples and unknown samples.

The ''Lack of Fit'' test was used to confirm the linearity of the method. Moreover, the back-calculated concentrations (C_{TEST}) were compared to the theoretical concentrations (C_{REF}), and the bias (or mean predictor error) was computed as follows:

$$\mathrm{Bias} = \frac{1}{n}\sum_{i=1}^{i=n}\left[C_{\mathrm{TEST}(i)} - C_{\mathrm{REF}(i)}\right]$$

The 95% confidence interval for bias was also computed.

2.6. Specificity

The specificity of the method was investigated by screening six different batches of blank human plasma and blood samples. The retention times of endogenous compounds in the matrix were compared with that of vinorelbine and internal standard.

Plasma and blood samples from patients receiving

other drugs were analyzed for interference. The following drugs were checked: amiodarone, alizapride, metoclopramide, zoplicone, methylprednisolone, paracetamol, dextropropoxyphene, dalteparin, clorazepate, ranitidine, omeprazole, furosemide, tamoxifene, amoxicilline, clavulanic acid, phenofribate, morphine.

2.7. Precision and accuracy

The precision and accuracy of the method were evaluated by performing replicate analyses of QC samples in plasma and blood against a calibration curve. Each QC sample was analyzed six-fold on the same day to determine intra-day precision and accuracy, and once a day during successive days (plasma, $n=12$; blood, $n=8$) to determine inter-day precision and accuracy.

Accuracy was expressed as the recovery, [mean back-calculated concentrations/theoretical concentrations]$\times$100, while the precision was given by the inter- and intra-day relative standard deviations (RSDs).

2.8. Effect of dilution

In order to test whether it is possible to apply the described method to samples whose concentrations are higher than the last calibration point, spiked samples at 900 ng/ml were prepared in plasma and blood. They were diluted 10- and 100-fold with blank human blood and 10-fold with blank human plasma in order to bring concentration within the range of standard curve. Each analysis was performed six times using calibration curves and QC samples. The found concentrations were reported and compared to the nominal ones.

2.9. Determination of the limits of quantitation and detection

The LOQ was defined as the lowest drug concentration which can be determined with an accuracy of 80–120% and a precision $\leq$20% on a day-to-day basis [19–21].

The LOD was defined as the sample concentration resulting in a peak area of three-times the noise level.

2.10. Extraction recovery

The extraction efficiency (recovery) was determined three times at QC concentration levels for vinorelbine and at the concentration used during the assay for the internal standard (50 ng/ml). The peak areas obtained after extraction were compared with peaks resulting from standard solutions in phosphate buffer at pH 2.5 at the same concentrations.

2.11. Stability study

The stability of stock solutions was tested at -20°C. The stability of working solutions was assessed at 20°C.

For stability studies in the two matrices, QC samples were used. The short-term stability was assessed after 1, 2, 4, 24 and 54 h of storage, under both ordinary laboratory conditions (20°C at daylight exposure) and at 4°C. The stability of the drug in frozen samples (-20°C) was determined by periodic analysis over 2 months. QC samples were analyzed immediately after preparation (i.e., fresh control samples prepared on the same day as the stability samples, reference values) then after storage. Prior to their analysis, frozen samples were brought to room temperature and vortex-mixed well. For each QC sample, three extractions were performed and concentrations were determined against a calibration curve.

The freeze–thaw stability was also determined. QC samples were analyzed immediately after preparation (reference values) and on a daily basis after repeated freezing–thawing cycles at -20°C on 2 consecutive days. Their concentrations were determined against a calibration curve.

The stability of vinorelbine in the acidic aqueous extracts was inspected after 12 h at 20°C and 24 h at 4°C.

A drug was considered stable if more than 90% of the intact drug was retained at the end of the study period.

3. Results

3.1. Retention times and specificity

Observed retention times were 5.4 and 4.5 min for vinorelbine and internal standard, respectively. The corresponding k' values were 1.83 and 1.38. There was clear resolution between the two drugs, separation factor = 2. The time intervals, where vinorelbine and the internal standard eluted, were free of interferences in all of the drug-free plasma and blood samples tested (Figs. 1a and 2a). No interference was found with the desacetylvinorelbine and with all drugs (and their metabolites) tested that could be co-administered.

Representative chromatograms are shown in Figs. 1 and 2.

3.2. Linearity

Peak area ratios of vinorelbine over the internal standard varied linearly with concentration over the range used. The ''Lack of Fit'' test showed no significant deviation from linearity. The determination coefficients (r^2) for calibration curves were equal to or better than 0.996. Inter-day repeatability was determined for calibration curves prepared on different days in plasma ($n = 12$) and blood ($n = 8$). Results are given in Table 1.

For each point of calibration standards, the concentrations were back-calculated from the equation of the linear regression curves (experimental concentrations) and the RSD values were computed. Inter-day variability at concentrations of calibration standards is presented in Table 2. From 2.5 to 100 ng/ml in plasma and from 5 to 100 ng/ml in blood, RSD values did not exceed 15% (2.3 to 15%); at the lowest concentrations (1 ng/ml in plasma; 2.5 ng/ml in blood), they were 12.7 and 20.5%, respectively. The recoveries ranged from 94 to 117%. A linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope and an intercept equal to 0 (Student's *t*-test). The distribution of the residuals (difference between nominal and back-calculated concentrations) shows random variations, the number of positive and negative values being approximately equal. They were normally distributed and centered around zero. The bias values ($-2.88 \cdot 10^{-4}$ for plasma; $-1.66 \cdot 10^{-9}$ for blood) were not statistically different from zero (Student's *t* test) and the 95% confidence intervals (−0.32–0.32 for plasma; −0.51–0.51 for blood) included the zero value.

3.3. Precision and accuracy

The results for accuracy, intra- and inter-day precision for QCs are presented in Table 3.

3.4. Extraction recovery

In plasma, the mean recovery ($n = 12$) averaged 101±5.3% for vinorelbine, it was 91.1±2.3% ($n = 12$) for the internal standard. In blood ($n = 12$), recoveries were 74.9±2.3 and 80.0±4.3% for the two analytes, respectively. The extraction efficiency was not statistically different over the range of concentrations studied.

3.5. Influence of the dilution

Dilution had no influence on the performance of the method which can be used up to 1800 ng/ml. At a theoretical concentration of 900 ng/ml, back-calculated concentrations averaged 812.9 ng/ml (precision, 4.7%) in plasma, and 844.5 (precision, 7.5%) and 885 ng/ml (precision, 5.6%) for the two dilutions studied (1/10 and 1/100) in blood, respectively. Accuracy values ranged from 92.3 to 98.3%.

3.6. Limit of quantitation and limit of detection

The LOQs were 1 and 2.5 ng/ml in plasma and blood, respectively. The LODs were 0.5 and 1 ng/ml in plasma and blood, respectively.

3.7. Stability

Stock solutions of vinorelbine and internal standard did not reveal any appreciable degradation after 6 months of storage at −20°C. At 20°C, working solutions of vinorelbine and vinblastine were stable for 20 min.

In plasma samples stored at 20 and 4°C during 54

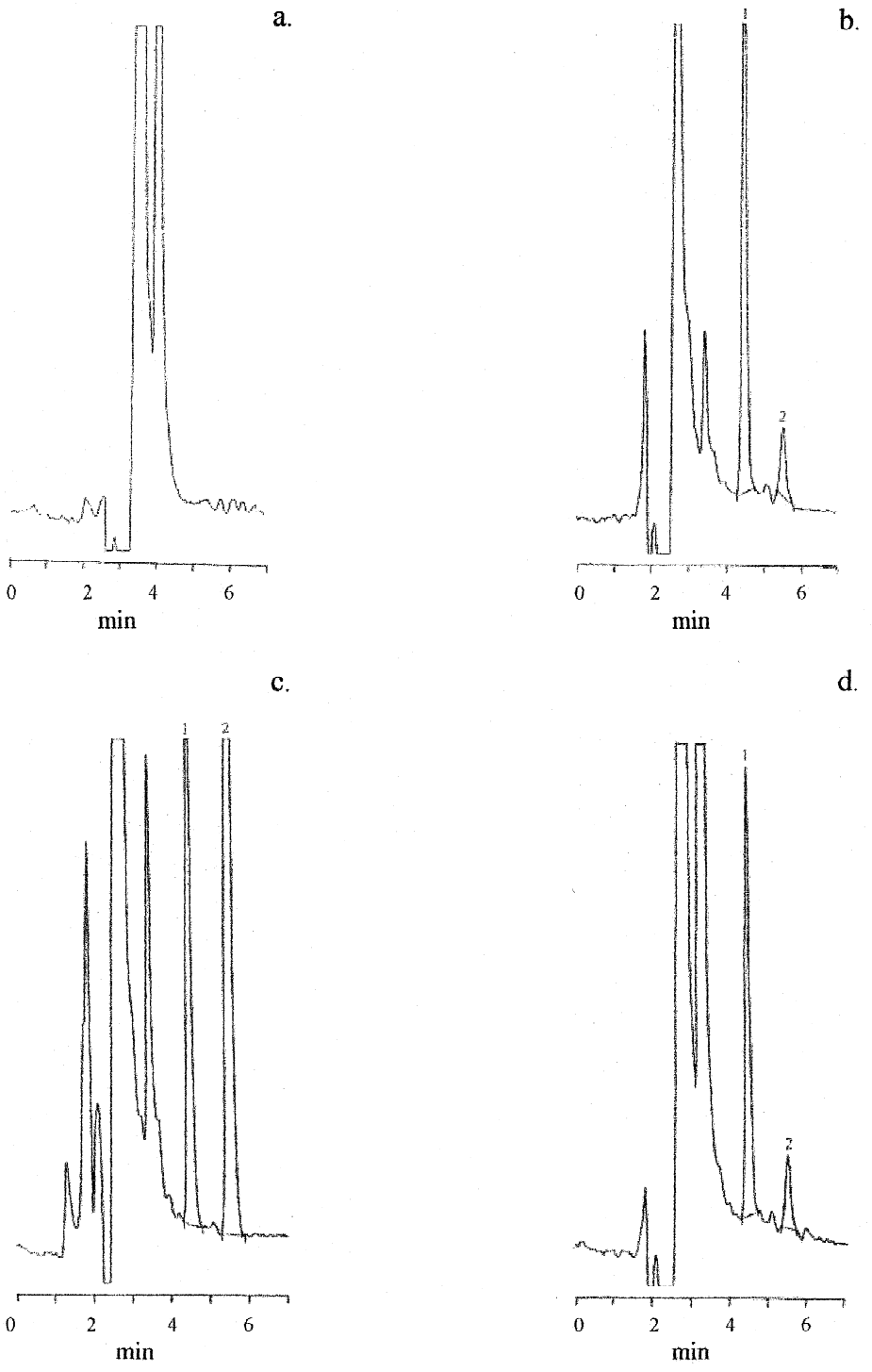

Fig. 1. Chromatograms of blank plasma (a). Plasma spiked with 2.5 ng/ml of vinorelbine (b). Plasma spiked with 50 ng/ml of vinorelbine (c). Plasma of a patient treated with 20 mg/m^2 of vinorelbine (plasma concentration: 2.95 ng/ml, 48 h following drug administration) (d). Peak 1 is vinblastine (internal standard), peak 2 is vinorelbine. For chromatographic conditions see Section 2.3.

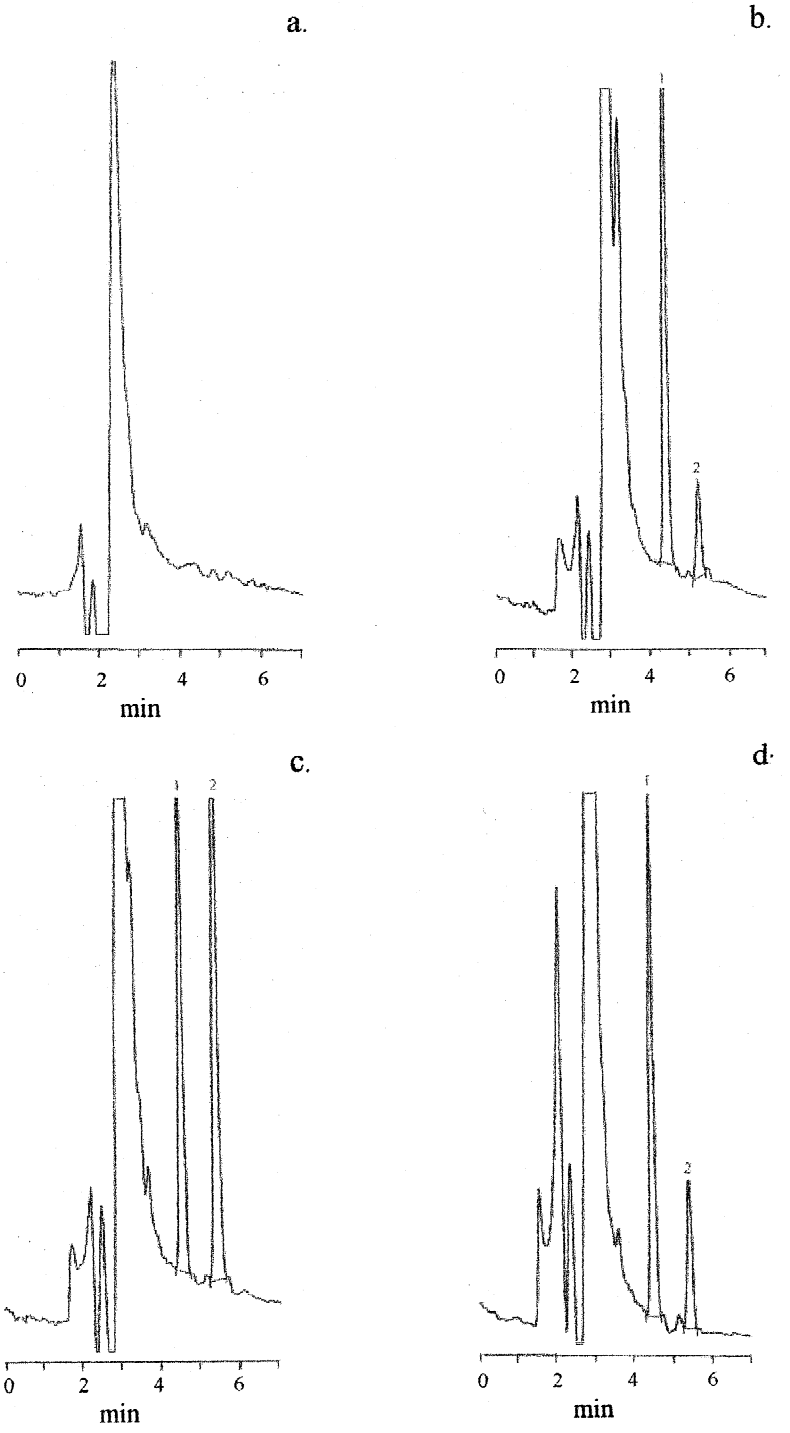

Fig. 2. Chromatograms of blank blood (a). Blood spiked with 5 ng/ml of vinorelbine (b). Blood spiked with 50 ng/ml of vinorelbine (c). Blood of a patient treated with 20 mg/m^2 of vinorelbine (blood concentration: 10.7 ng/ml, 24 h following drug administration) (d). Peak 1 is vinblastine (internal standard), peak 2 is vinorelbine. For chromatographic conditions see Section 2.3.

Table 1
Assay linearity of the method

	Determination coefficient of the linear regression analysis[a] ($r^2 \pm$SD)	*a*, slope±SD	*b*, intercept±SD
Plasma			
Inter-day reproducibility ($n=12$)	$0.998 \pm 9.7 \cdot 10^{-4}$ RSD=0.097%	$4.65 \cdot 10^{-3} \pm 7.7 \cdot 10^{-3}$ RSD=16.6%	−0.0314±0.029
Blood			
Inter-day reproducibility ($n=8$)	$0.997 \pm 1.1 \cdot 10^{-3}$ RSD=0.11%	$2.9 \cdot 10^{-2} \pm 2.82 \cdot 10^{-3}$ RSD=9.7%	0.0035±0.023

[a] Linear unweighted regression, formula: $y=ax+b$.
r^2=Determination coefficient. n=Number of replicates.

h, vinorelbine did not reveal any appreciable degradation, with all samples retaining more than 92% of their original concentration values. In blood samples, vinorelbine was stable 6 h at 20 and 4°C.

The long-term freezer stability indicated that vinorelbine was stable during 2 months, the recovery was higher than 90%. Compared to the reference values, no statistical difference appeared.

Run-time stabilities of acidic aqueous extracts at 20 and 4°C were determined for each point of the calibration standard. After 12 and 24 h no significant losses occurred, respectively.

At least two freeze–thaw cycles can be tolerated without losses higher than 10%.

3.8. Pharmacokinetic study

This overall analytical procedure has been used to determine concentrations of vinorelbine in plasma and blood samples from patients aged 65 years or

Table 2
Back-calculated concentrations from calibration curves

Theoretical concentration (ng/ml)	Back-calculated concentration (ng/ml)	RSD (%)	Recovery (%)
Plasma ($n=12$)			
1	1.17	12.7	117.0
2.5	2.87	12.8	114.8
5	5.14	9.06	102.8
10	9.88	5.64	98.8
25	24.5	3.75	97.9
50	49.4	4.15	98.7
80	79.0	3.09	98.7
100	101.3	2.28	101.3
Blood ($n=8$)			
2.5	2.35	20.5	94.2
5	5.39	14.9	107.8
10	9.80	11.7	98.0
25	24.6	4.52	98.4
50	50.6	3.57	101.2
80	80.5	4.22	100.6
100	99.4	3.12	99.4

n=Number of replicates.

Table 3
Assessment of the accuracy and precision of the method

Theoretical concentration (ng/ml)	Back-calculated concentration (ng/ml)	RSD (%)	Recovery (%)
Plasma			
Intra-day ($n=6$)			
7.5	7.90	6.14	105.4
20	18.9	3.86	94.5
40	40.8	5.70	102.0
Inter-day ($n=12$)			
1	1.20	15.6	119.9
7.5	7.63	13.5	101.7
20	19.7	8.49	98.3
40	41.5	9.32	103.8
Blood			
Intra-day ($n=6$)			
6.5	5.95	8.14	91.6
20	19.1	11.0	95.6
35	34.1	7.66	97.5
Inter-day ($n=8$)			
2.5	2.68	19.6	107.2
6.5	6.78	15.8	104.3
20	18.8	11.0	94.2
35	34.2	12.4	97.8

n = Number of replicates.

older, with metastatic cancer in progression. The study protocol was reviewed and approved by the institutional review board. Vinorelbine was administered by a 10-min continuous infusion at a dose of 20–30 mg/m^2 through a central venous catheter. Serial blood samples were collected from a peripheral vein in heparinized glass tubes before drug administration, at the end of infusion, and 20 min, 1, 6, 12, 18, 24, 48 and 72 h after the start of infusion. Immediately after collection plasma was obtained by centrifugation (1500 *g* for 10 min) at 4°C. Pharmacokinetic parameters were determined using the pK-fit software [22]. Fig. 3 shows the plasma and blood concentration versus time profiles of vinorelbine in a representative patient. The elimination half-life was of the same order of magnitude for plasma and blood, about 42 h. The ratio AUC_{blood}/AUC_{plasma} [AUC being the area under plasma (or blood) concentration versus time curve] was 1.6. It was comparable to the blood/plasma ratio of this patient, 1.6 that remained constant over the 72 h of the study.

4. Discussion and conclusion

In this manuscript, we developed and validated a sensitive and reproducible HPLC method with fluorescence detection to quantify vinorelbine in human plasma and blood samples. To our knowledge, it is the first validated method allowing the quantification of vinorelbine in blood. As vinorelbine is 84% bound to blood cells, mainly to platelets [18], it seems important to determine blood concentrations of this drug. The present method is relatively easy to perform and allows to determine vinorelbine in blood and plasma at the nanogram level. Compared with fluorescence detection, scintigraphic and electrochemical detections are also very sensitive but difficult to handle. In therapeutic drug monitoring, fluorescence detection is certainly the best routinely usable detection mode for drugs showing sufficient fluorescence properties, such as vinorelbine. Assay performance of the present method was assessed both on the basis of the statistical characteristics of individual calibration curves and from the results of

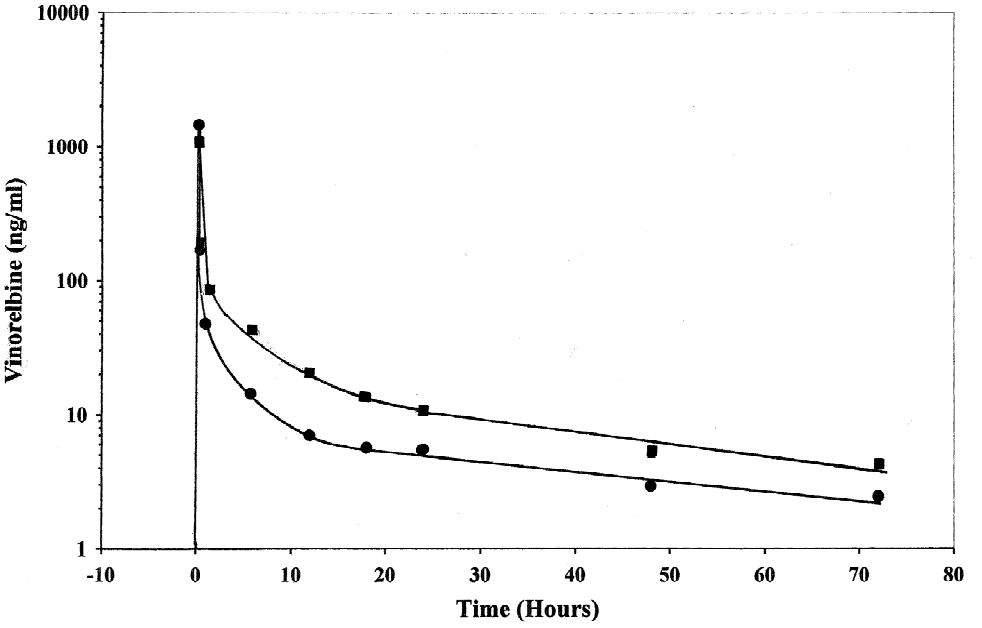

Fig. 3. Plasma (●) and blood (■) concentration–time profiles of vinorelbine after a 10-min intravenous infusion of 20 mg/m^2 of the drug to a patient with breast cancer.

QC samples. Vinblastine was regarded as an acceptable internal standard because it exhibits similar extraction properties; moreover, its use allowed enhanced precision. The LOQs were 1 and 2.5 ng/ml in plasma and blood, respectively. In plasma, the LOQ reported in the present study was similar to that published by Nicot et al. [12] but lower than the other previously published methods. The present method validation results indicate that the performance characteristics of the method fulfilled the requirements for a sufficiently accurate and precise assay method to carry out pharmacokinetic studies. The applicability of this assay was demonstrated in a pharmacokinetic study in a patient suffering from metastatic cancer in progression, receiving 20 mg/m^2 vinorelbine as a 10-min intravenous infusion.

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