Journal of Chromatography, 581 (1992) 93–99
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6463

Determination of vinorelbine (Navelbine) in tumour cells by high-performance liquid chromatography

V. Debal, H. Morjani, J.-M. Millot and J.-F. Angiboust

Laboratoire de Spectroscopie Biomoléculaire, U.F.R. de Pharmacie, 51 Rue Cognacq Jay, 51096 Reims Cedex (France)

B. Gourdier

Laboratoire de Pharmacie Clinique, Pharmacie Centrale, Hôpital Robert Debré, C.H.R. Reims, 51092 Reims Cedex (France)

M. Manfait

Laboratoire de Spectroscopie Biomoléculaire, U.F.R. de Pharmacie, 51 Rue Cognacq Jay, 51096 Reims Cedex (France)

(First received January 14th, 1992; revised manuscript received May 19th, 1992)

ABSTRACT

A high-performance liquid chromatographic method has been developed for the determination within tumour cells of a new vinca alkaloid, vinorelbine. Extractions of vinorelbine from cells were carried out using absolute ethanol. The extracts were injected into a reversed-phase system consisting of two Novapak C_{18} columns connected in series. The mobile phase was acetonitrile–phosphate buffer, pH 2.7 (60:40, v/v). Using a fluorescence detection, the limit of determination was 8 pmol injected. This method would be suitable for studying the cellular pharmacokinetics of vinorelbine in patients.

INTRODUCTION

Vinorelbine (Navelbine), a new hemisynthetic vinca alkaloid, proved to be of interest in the treatment of non-small cell lung cancers, where it showed an improved activity compared with other vinca alkaloids [1]. Its efficacy against other types of cancer, and especially breast cancer, has also been demonstrated [2].

Several techniques have been published for the high-performance liquid chromatographic (HPLC) determination of vinorelbine [3–5] and other vinca alkaloids [6–8] in plasma and urine. However, there are few methods for the determination of vinca alkaloids within tumour cells [8,9] and none of these involves vinorelbine.

This paper describes a simple and rapid method for the reversed-phase HPLC determination of vinorelbine in tumour cells, with fluorescence detection.

Correspondence to: Professor Michel Manfait, Laboratoire de Spectroscopie Biomoléculaire, U.F.R. de Pharmacie, 51 Rue Cognacq Jay, 51096 Reims Cedex, France.

EXPERIMENTAL

Chemicals and reagents

Vinorelbine bitartrate, desacetylvinorelbine (one of its metabolites) and [^{3}H]vinorelbine (0.68 Ci/mmol) were obtained from Pierre Fabre Médicaments (Paris, France) and vinblastine (Velbe) from Eli Lilly (St. Cloud, France). Aqueous solutions of vinorelbine and vinblastine (10^{-3} *M*)

0378-4347/92/$05.00 © 1992 Elsevier Science Publishers B.V. All rights reserved

were prepared, divided into 200-μl aliquots and stored at −20°C. Under these conditions, the solutions were stable for at least three months. Acetonitrile (for HPLC), potassium dihydrogenphosphate, absolute ethanol and sulphuric acid were obtained from Prolabo (Paris, France). Phosphoric acid was supplied by Farmitalia (Milan, Italy). Sodium dodecyl sulphate was obtained from Sigma (La Verpillière, France).

Cell cultures

K562 is a human myeloid leukaemia cell line [10]. Cell suspensions were grown at concentrations ranging from 10^5 to 10^6 cells per ml of RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum and 2 m*M* L-glutamine.

The resistant cell variants (K562-R), cross-resistant to anthracyclines, vinca alkaloids and other antineoplastic drugs, were obtained by continuous exposure to increasing concentrations from 10 to 100 n*M* of doxorubicin over a period of eight months and then maintained at a constant concentration of 100 n*M*. This subline expresses a membrane glycoprotein (GP170) and possesses amplified DNA sequences responsible for the multi-drug resistance phenotype [11].

Incubations were performed at 37°C in a CO_2 incubator. Samples of $4 \cdot 10^6$ cells were incubated in 8 ml of RPMI 1640 medium containing vinorelbine for different time periods: 0.5, 1, 2, and 3 h. Cell densities and viability were determined by phase contrast microscopy with 0.1% trypan blue.

Extraction of vinorelbine from K562 cells

After incubation in the presence of vinorelbine, the cell suspensions were centrifuged for 10 min at 200 *g*. Supernatants were discarded and pellets were washed twice with 2 ml of phosphate-buffered saline, then 20 μl of a 10^{-5} *M* vinblastine solution (internal standard) were added to each sample. Extraction was performed using ethanol acidified to pH 5.5 with sulphuric acid. A 200-μl volume of this ethanol solution was added to each sample. Tubes were shaken for 2 min on a vortex mixer and centrifuged for 10 min at 3000 *g*. A 25-μl volume of each supernatant was then directly injected into the chromatograph.

Chromatography

The chromatographic system consisted of a Shimadzu LC7A solvent-delivery module (Touzart et Matignon, Vitry-sur-Seine, France), a U6K injector (Waters, St. Quentin en Yvelines, France) and a Shimadzu RF 530 fluorescence detector (Touzart et Matignon) set at an excitation wavelength of 280 nm and an emission wavelength of 360 nm. A Waters 484 absorbance detector (set at a wavelength of 268 nm) was used for comparison. Chromatograms were recorded and peaks were integrated using a computer with a specially developed software. Separations were performed with two columns in series: a Novapak C_{18} column (300 mm × 3.9 mm I.D.; 4 μm particle size) and a Novapak C_{18} column (150 mm × 3.9 mm I.D.; 4 μm particle size) (both Waters).

The mobile phase was acetonitrile–25 m*M* phosphate buffer (pH 2.7 adjusted with phosphoric acid) (60:40, v/v) containing 0.1 g/l sodium dodecyl sulphate. The flow-rate was 0.9 ml/min.

Effect of the mobile phase composition

In order to optimize the fluorescence yield and retention time of vinorelbine, the effects of the pH and the acetonitrile concentration in the mobile phase were tested.

Vinorelbine solutions (1 μ*M*) were prepared in acetonitrile–25 m*M* phosphate buffer (60:40, v/v) and adjusted to pH values in the range 7–2 with phosphoric acid. Also, 1 μ*M* vinorelbine solutions were prepared in acetonitrile–25 m*M* phosphate buffer (pH 2.7) with acetonitrile concentrations from 0 to 100% (v/v). Fluorescence spectra of these solutions were recorded using a Shimadzu RF 5000 spectrofluorophotometer.

The capacity factors of vinorelbine, desacetylvinorelbine and vinblastine were determined with mobile phase adjusted to pH 2.5, 3, 3.5 and 4, and also with concentrations of acetonitrile in the mobile phase ranging from 55 to 70%.

Calibration curves

Intracellular vinorelbine amounts were determined from calibration curves established with

six standard solutions of known concentrations: 6, 10, 25, 50, 75 and 100 μM. Six pellets of untreated K562 cells were spiked with 20 μl of the vinorelbine standard solutions and 20 μl of the internal standard. After extraction and HPLC analysis as described above, peak-area ratios (vinorelbine/internal standard) were plotted against the corresponding vinorelbine amounts in each standard solution (0.12, 0.2, 0.5, 1, 1.5 and 2 nmol).

Extraction recovery

To determine the vinorelbine extraction recovery, K562 cells were incubated with 0.2 and 2 μM [^{3}H]vinorelbine for 1 h. [^{3}H]Vinorelbine extraction was performed on half of the cell samples. The radioactivity of each cell sample or sample extract was determined using an Intertechnique liquid scintillation counter (Kontron Instruments, Montigny-le-Bretonneux, France) after the addition of 4 ml of a liquid scintillation cocktail (Beckman Instruments, Gagny, France).

To determine the extraction recoveries of vinblastine and desacetylvinorelbine, six cellular pellets were spiked with 50, 200 and 500 pmol of vinblastine or 100, 500 and 1000 pmol of desacetylvinorelbine. The extraction and HPLC determination were performed as described above.

RESULTS

Detection mode

In the mobile phase, the excitation optimum of vinorelbine was found to be 280 nm and its emission optimum to be 360 nm. These wavelengths proved to be the same for vinblastine but are slightly different for vincristine.

Vinca alkaloids show great differences in fluorescence intensity from one compound to another [7]. For example, the fluorescence emission intensity of vinorelbine, measured in the mobile phase, was three times less than that of vinblastine, but five times greater than that of vincristine. Thus, under our experimental conditions, fluorescence proved to be a suitable detection mode for vinorelbine and vinblastine determination, but not for vincristine determination.

Chromatography

Among the stationary phases used for vinca alkaloid determination, C_{18} bonded phases [4,6,8] and cyano bonded phases appear to be the most commonly used. Thus, we first tried a Novapak C_{18} (150 mm × 3.9 mm I.D.) column. However, although this column clearly resolved vinorelbine and vinblastine (the internal standard), it did not separate vinblastine from desacetylvinorelbine. A longer Novapak C_{18} column (300 mm × 3.9 mm I.D.) gave a better, but still insufficient, resolution for the latter pair. The best separation was obtained with these two columns in series.

Fig. 1 shows chromatograms obtained from untreated and vinorelbine-treated K562 and K562-R cell extracts. The retention times were *ca.* 5.6 min for vinblastine, 6.6 min for desacetylvinorelbine and 9.2 min for vinorelbine.

Effect of mobile phase composition

The fluorescence intensity from vinorelbine increased with decreasing pH (Fig. 2A) and with increasing acetonitrile concentration (Fig. 2B) in the mobile phase.

Figs. 3 and 4 show the effects of the mobile phase composition (pH, percentage of acetonitrile) on the capacity factors (k') of vinorelbine, desacetylvinorelbine and vinblastine.

The final choice of pH (2.7) and of acetonitrile concentration (60%, v/v) was a compromise between detection limit and resolution.

Linearity

The linearity of the method was studied at different vinorelbine concentrations (6, 10, 25, 50, 75, 100, 150, 250 and 500 μM) using the same method as in the case of calibration curves. Peak-area ratios (vinorelbine/internal standard) were plotted against vinorelbine amounts in the standard solutions (0.12, 0.2, 0.5, 1, 1.5, 2, 3, 5 and 10 nmol). The correlation coefficient was 0.9976. The equation of the regression line was $y = 0.68x + 0.04$.

Reproducibility

The reproducibility was determined for K562

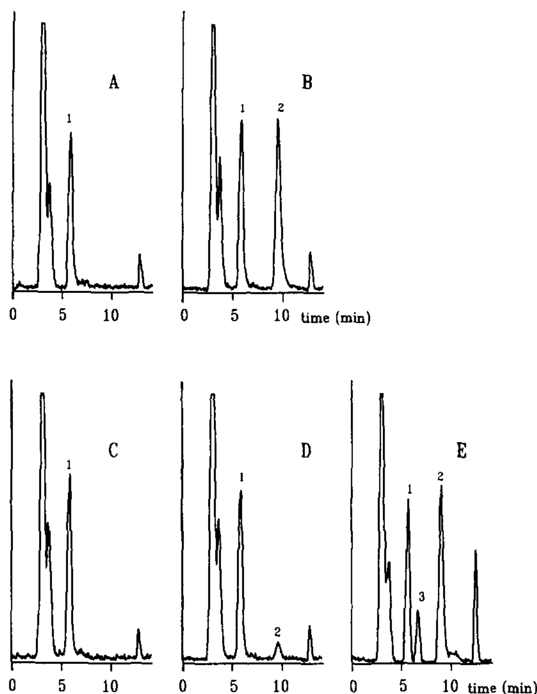

Fig. 1. Chromatograms of cell extracts. (A) Blank K562 cell extract (containing 200 pmol internal standard); (B) K562 cell extract after incubation for 1 h with 2 μM vinorelbine (corresponding to 78.4 pmol vinorelbine injected); (C) blank K562-R cells; (D) K562-R cell extract after incubation for 1 h with 2 μM vinorelbine (8.1 pmol vinorelbine injected); (E) K562 cell extract after incubation for 1 h with 2 μM vinorelbine and spiked with 200 pmol desacetylvinorelbine. Peaks: 1 = vinblastine; 2 = vinorelbine; 3 = desacetylvinorelbine.

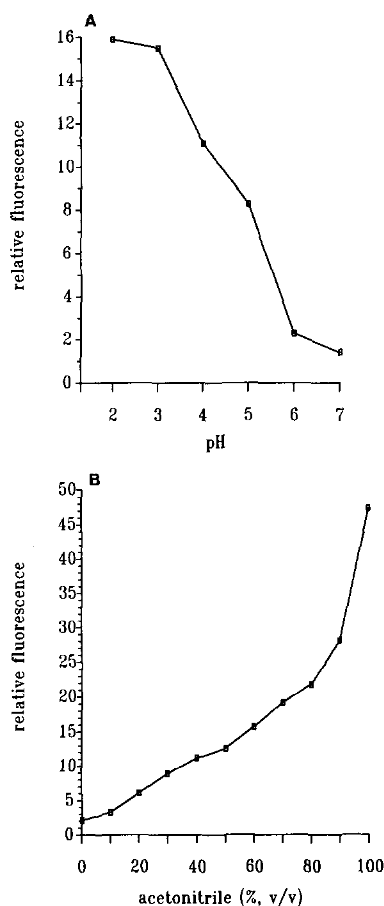

Fig. 2. Fluorescence intensity of 1 μM vinorelbine (A) as a function of pH (acetonitrile–phosphate buffer, 60:40, v/v) and (B) as a function of acetonitrile concentration (pH 2.7). Excitation wavelength, 280 nm; emission wavelength, 360 nm.

and K562-R cells incubated for 2 h with two different concentrations of vinorelbine. Six determinations were performed for each concentration. The inter-assay variability was obtained by carrying out five different incubations over five days. The coefficients of variation (C.V.) obtained for K562 cells incubated with 0.2 or 2 μM vinorelbine and for K562-R cells incubated with 2 or 10 μM vinorelbine are shown in Table I. The high inter-assay C.V. can be explained by differences in day-to-day cell culture conditions.

Extraction recoveries and detection limits

The extraction recoveries determined by scintigraphy after incubation of K562 cells with [^{3}H]vinorelbine, as described in Experimental,

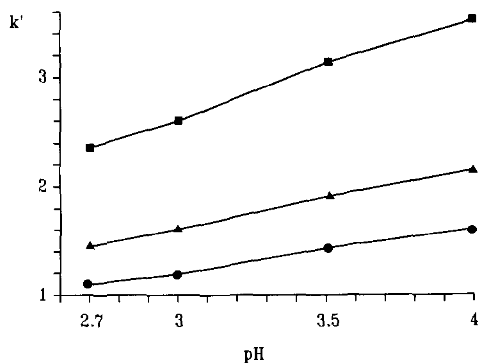

Fig. 3. Capacity factors of vinblastine (●), vinorelbine (■) and desacetylvinorelbine (▲) as a function of pH (acetonitrile–phosphate buffer, 60:40, v/v).

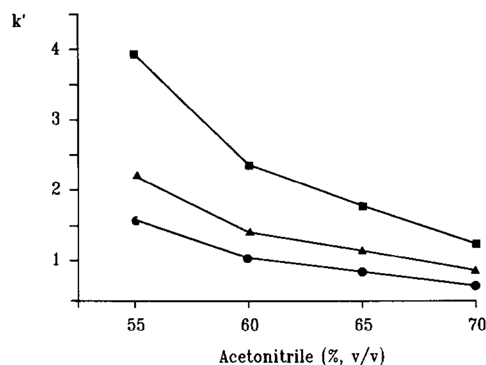

Fig. 4. Capacity factors of vinblastine (●), vinorelbine (■) and desacetylvinorelbine (▲) as a function of acetonitrile concentration (v/v) (pH 2.7).

were always greater than 90% (Table II). The extraction recoveries of vinblastine and desacetylvinorelbine determined by HPLC are shown in Table II.

The detection limits, defined as the amount of drug injected giving a signal-to-noise ratio of 3, were 8 pmol for vinorelbine and 2 pmol for vinblastine. These values were lower than those obtained with UV detection (268 nm), which were 13 pmol for vinorelbine and 12 pmol for vinblastine.

Selectivity

Several drugs were injected to evaluate possible interferences: antibiotics (amoxicillin, doxycycline, rifamycin), analgesics (acetylsalicylic acid, paracetamol, noramidopyrine), antitumour drugs (aclacinomycin, doxorubicin, daunorubicin, methotrexate), insulin and heparin. None of these drugs interfered in the determination of vinorelbine.

Cellular uptake of vinorelbine by K562 and K562-R cells

K562 and K562-R cells were incubated for 0.5, 1, 2 and 3 h in RPMI 1640 containing 2 μM vinorelbine. For each incubation time, three different samples were incubated and vinorelbine amounts were determined per 10^6 cells. The chromatograms (Fig. 1B and D) show the absence of intracellular metabolites such as desacetylvinorelbine. Results from Fig. 5 show that vinorelbine accumulates more in K562 than in K562-R cells (10.9-fold more after 2 h). In the light of data associating a membrane glycoprotein (GP170) with the resistance of K562-R cells, this result could be explained by a drug efflux out of these cells, which reduces the accumulation of drug in the cells [11,12].

DISCUSSION

Different methods have been employed for pharmacokinetic studies of vinca alkaloids. Some of them, using radioactive vinorelbine or radioimmunological techniques [13–15], have some drawbacks. These methods cannot distinguish the drug from its metabolites, and the use of ra-

TABLE I

REPRODUCIBILITY OF VINORELBINE INTRACELLULAR DETERMINATION

Cell type	Incubation concentration (μM)	Intra-assay (n = 6)		Inter-assay (n = 5)	
		Intracellular concentration (mean ± S.D.) (pmol/10^6 cells)	C.V. (%)	Intracellular concentration (mean ± S.D.) (pmol/10^6 cells)	C.V. (%)
K562	0.2	23 ± 2	10.1	24 ± 5	19.6
	2	214 ± 9	4.2	239 ± 39	16.3
K562-R	2	20 ± 2	11.9	23 ± 5	20.1
	10	94 ± 7	7.6	102 ± 15	15.0

TABLE II

EXTRACTION RECOVERIES

Drug	Incubation concentration or amount	Recovery (%)	*n*	C.V. (%)
[^{3}H]Vinorelbine	0.2 μM	96.3	4	5.1
	2 μM	99.1	4	4.7
Vinblastine	50 pmol	88.9	5	8.5
	200 pmol	93.7	5	3.4
	500 pmol	93.2	5	2.5
Desacetylvinorelbine	100 pmol	87.9	5	5.0
	500 pmol	94.4	5	3.6
	1000 pmol	90.5	5	2.8

dioactive elements in patients raises ethical problems. These problems can be solved using HPLC, a method applicable to routine studies. Several detection modes have been used in HPLC studies: scintigraphic [5], electrochemical [4,6], UV [3,8,9] and fluorescence [7] detection. Scintigraphy and electrochemical detection are very sensitive but difficult to handle. Compared with fluorescence detection, UV detection shows a lower selectivity. Moreover, some interferences could arise with biological samples. Fluorescence detection, which is more selective and easy to handle, is certainly the best routinely usable detection mode for molecules showing sufficient fluorescence properties, such as vinorelbine and vinblastine.

The rapid and simple method described here for *in vitro* studies could be suitable for cellular pharmacokinetic studies of vinorelbine in patients. Thus, the determination of antitumour drugs in patient tumour cells could provide data additional to those from determinations, in plasma and urine.

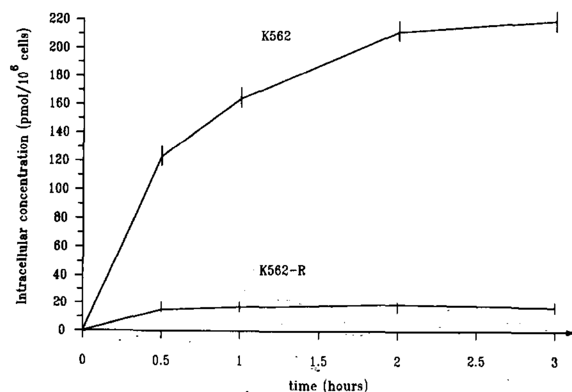

Fig. 5. Intracellular concentration of vinorelbine (pmol per 10^6 cells) in K562 and K562-R cells as a function of incubation time. Each point is the average of three independent determinations. Initial vinorelbine concentration in the medium, 2 μM.

ACKNOWLEDGEMENTS

This work was supported by Laboratoires Pierre Fabre Médicament (Castres, France). We thank Dr. Denis Bosson for his helpful English revision of our manuscript.

REFERENCES

1 A. Depierre, E. Lemarie, G. Dabouis, G. Garnier, P. Jacoulet and J. C. Dalphin, *Semin. Oncol.*, 16 (Suppl. 4) (1989) 26.
2 L. Canobbio, F. Boccardo, G. Pastorino, F. Brema, C. Martini, M. Resasco and L. Santini, *Semin. Oncol.*, 16 (Suppl. 4) (1989) 33.
3 F. Jehl, J. Debs, C. Herlin, E. Quoix, C. Gallion and H. Monteil, *J. Chromatogr.*, 525 (1990) 225.
4 G. Nicot, G. Lachatre, P. Marquet, F. Bonnaud, J. P. Valette and J. L. Rocca, *J. Chromatogr.*, 528 (1990) 258.
5 P. Bore, R. Rahmani, J. Van Canfort, C. Focan and J. P. Cano, *Cancer Chemother. Pharmacol.*, 23 (1989) 247.

6 D. E. M. M. Vendrig, J. Teeuwsen and J. J. M. Holthuis, *J. Chromatogr.*, 424 (1988) 83.

7 D. E. M. M. Vendrig, J. Teeuwsen and J. J. M. Holthuis, *J. Chromatogr.*, 434 (1988) 145.

8 J. A. Houghton, P. M. Torrance and P. J. Houghton, *Anal. Biochem.*, 134 (1983) 450.

9 M. De Smet, S. J. P. van Belle, V. Seneca, G. A. Storme and D. L. Massart, *J. Chromatogr.*, 416 (1985) 375.

10 C. B. Lozzio and B. B. Lozzio, *Blood*, 45 (1975) 321.

11 T. Tsuruo, H. Iida, H. Kawataba, T. Oh-hara, H. Hamada and T. Utakoji, *Jpn. J. Cancer Res.*, 77 (1986) 682.

12 N. Kartner, J. R. Riordan and V. Ling, *Science*, 221 (1983) 1285.

13 R. Rahmani, R. Bruno, A. Iliadis, R. Faure, S. Just, J. Barbet and J. P. Cano, *Cancer Res.*, 47 (1987) 5796.

14 R. L. Nelson, R. W. Dyke and M. A. Root, *Cancer Treat. Rev.*, 7 (Suppl.) (1980) 17.

15 R. J. Owellen, C. A. Hartke and F. O. Hains, *Cancer Res.*, 37 (1977) 2597.