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Note

High-performance liquid chromatographic determination of navelbine in human plasma and urine

G. NICOT*, G. LACHATRE and P. MARQUET

Département de Pharmacologie Clinique, Centre Hospitalier Régional Universitaire, Hôpital Dupuytren, 87042 Limoges (France)

F. BONNAUD

Service de Pathologie Respiratoire, Centre Hospitalier Régional Universitaire, Hôpital du Cluzeau, 87042 Limoges (France)

J P. VALLETTE

Département de Pharmacologie Clinique, Centre Hospitalier Régional Universitaire, Hôpital Dupuytren, 87042 Limoges (France)

and

J.-L. ROCCA

Laboratoire des Sciences Analytiques, U.A. CNRS 040435, J.-M. Mermet, Université Claude Bernard, Villeurbanne (France)

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Navelbine (5′-noranhydrovinblastine), recently developed by Mangeney and co-workers [1,2], is a potential alternative to natural or semisynthetic vinca alkaloids. It has shown anti-neoplastic properties in experimental systems and a possible absence of cross-resistance with vincristine [3,4]. In order to study the pharmacokinetics of this drug and to provide information necessary for its clinical development, a specific and reliable method for the determination of this compound in biological fluids was required. Up to now, only a radioimmunoassay technique has been published [5] and applied to the phase I study [6] and to preliminary pharmacokinetic evaluation [5,7,8].

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This paper describes a simple high-performance liquid chromatographic (HPLC) method for plasma and urine determination of navelbine. This method, which is performed using an internal standard, a reversed-phase ion-pair system and amperometric detection, is suitable for pharmacokinetic investigations and assays on a routine basis in humans.

EXPERIMENTAL

Chemicals and reagents

Navelbine bitartrate and vinblastine sulphate (internal standard) were kindly provided by Pierre Fabre Labs. (Paris, France) and Lilly-France Labs. (Saint-Cloud, France), respectively. Methanolic solutions (1 mg ml^{-1}) of navelbine and the internal standard were prepared and stored in glass containers at $-20°C$; they were stable for at least one month. A 10 μg ml^{-1} solution of navelbine and a 1 μg ml^{-1} solution of internal standard were prepared weekly by diluting the respective standard solutions with methanol. Diethyl ether (Normapur grade), methanol (for reversed-phase HPLC), hexane (for HPLC) and sodium dihydrogenphosphate dihydrate (pro analysis) were all obtained from Prolabo (Paris, France). Ethyl acetate (HPLC grade) and acetonitrile (HPLC grade) were purchased from Rathburn (Cluzeau Info Labo, Sainte-Foy-la-Grande, France). Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and heptanesulphonic acid sodium salt were provided by Fluka (Labex, Mulhouse, France) and Eastman Kodak (Touzart et Matignon, Vitry-sur-Seine, France), respectively.

Chromatography

The chromatographic apparatus consisted of the following components: a Chromatofield Model 501 pump (Chromatofield, Chateauneuf-les-Martigues, France), a Rheodyne 71-25 injection valve (Touzart et Matignon) equipped with a 120-μl loop and a Chromatofield Model Eldec 103 electrochemical detector. This detector was operated in the amperometric mode at 0.93 V with a glassy carbon working electrode and an Ag/AgCl reference electrode. The detector output was connected to a Kipp & Zonen Model BP 40 recorder or a Shimadzu Model C-R3A integrator (Touzart et Matignon). A stainless-steel column (150 mm × 4.6 mm I.D.) was packed with Nucleosil C_{18} 5-μm stationary phase (Macherey-Nagel, Düren, F.R.G.) using a slurry packing technique [9] with modifications to the solvent used: the slurry was made with *n*-butanol and the packing solvent was methanol.

The mobile phase consisted of acetonitrile–methanol–0.1% (w/v) aqueous sodium dihydrogenphosphate (8:50:42, v/v/v). Aqueous sodium dihydrogenphosphate solution contained 0.4 g l^{-1} heptanesulphonate and 0.3 g l^{-1} EDTA, and was adjusted to pH 3.0 with 1 *M* phosphoric acid. This mobile phase was filtered using a 0.45-μm Millipore filter and degassed in an ultrasonic bath. A

flow-rate of 1.1 ml min^{-1} was maintained, and the separation was performed isocratically at room temperature.

Sample collections

Venous blood samples (5 ml) were collected into 10-ml Vacutainer green-stoppered tubes (Becton-Dickinson, Missisauga, Canada) and centrifuged as soon as possible at 900 *g*. The volume of urine samples was recorded after collection. When the determination was not carried out immediately, plasma and urine samples were frozen at −20 °C in polystyrene tubes. Although the stability of navelbine in plasma and urine was not investigated, we could detect no degradation when these biological samples were kept at −20 °C in polystyrene tubes for at least one month, according to results obtained by De Smet et al. [10] with vinblastine.

Extraction from biological samples

Extraction from plasma. For analysis, 1 ml of plasma was added to 100 µl of 1 µm ml^{-1} internal standard solution and 8 ml of diethyl ether in 25-ml PTFE-lined screw-capped glass tubes. The tubes were shaken for 10 min on a Laboral oscillating agitator (Prolabo), centrifuged at 900 g for 10 min and frozen at −20 °C for 45 min. A 7-ml volume of the upper organic phase was collected in a 10-ml conical glass tube, and evaporated to dryness under a very gentle nitrogen stream at 35–40 °C. The dry residue was redissolved in 250 µl of mobile phase and washed twice with 1 ml of hexane. A 100-µl aliquot of this aqueous solution was injected into the chromatograph or kept at +4 °C before injection.

Extraction from urine. After urine was centrifuged at 900 *g* to remove particulates, the extraction procedure was similar to that for plasma, except that 500 µl of 0.1 *M* sodium dihydrogenphosphate (pH 7.0) were added to each sample before analysis.

Calibration curves and calculation

Calibration graphs were constructed to demonstrate the linear relationship between the peak-height ratio and the concentration of the samples. The amounts of navelbine used for the establishment of the standard curves were of the same magnitude as those found in biological samples (our study): the range covered 1–1000 ng ml^{-1} for plasma and 20–1000 ng ml^{-1} for urine. The spiked calibration samples were submitted to the extraction procedure described previously and standard curves were generated by plotting peak-height ratios (drug/internal standard) against six known drug concentrations when using the Kipp & Zonen recorder. Plasma and urine concentrations of navelbine were interpolated from these standard curves.

RESULTS AND DISCUSSION

Chromatographic separation

Typical chromatograms of extracts from blank human plasma, patient plasma and urine are shown in Fig. 1. The applied HPLC method should be very selective for the monitoring of plasma and urine samples because cancer patients often received extensive medication (antibiotics, anti-emetics, analgesics, etc.), which can interfere with this analysis. To take this risk into account, high selectivity was generally expected using reversed-phase ion-pair partition chromatography. Owing to the pK_a values of navelbine (5.4) and internal standard (5.4 and 7.4) and the nitrogen atoms in their molecules, these drugs are protonated when the pH of the mobile phase is adjusted to 3.0. Hence they can be chromatographed in presence of an anionic counter-ion such as heptanesulphonate.

Several acetonitrile– or methanol–buffer mixtures were investigated as possible mobile phases. The best results were obtained with the mixture described

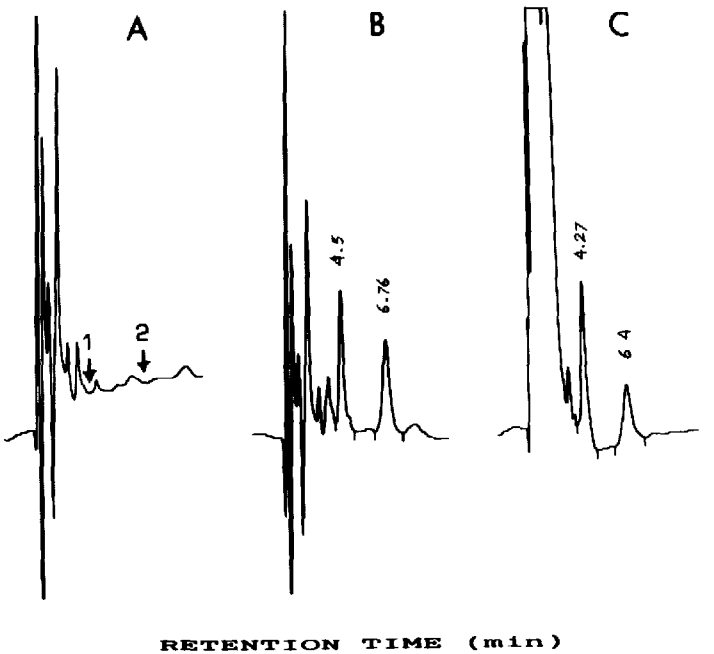

Fig. 1. Typical chromatograms of extracts from: (A) blank plasma (pre-dose patient plasma); the arrows 1 and 2 show the absence of peaks at the retention times of internal standard and navelbine, respectively; (B) a plasma sample obtained from a patient 2 h after receiving a 30 mg m^{-2} infusion of navelbine, containing 110 ng ml^{-1} (6.76 min) of this drug and spiked with 100 ng ml^{-1} internal standard (4.5 min); (C) a urine sample obtained from a patient 72 h after receiving a 30 mg m^{-2} infusion of navelbine, containing 39 ng ml^{-1} of this drug (6.4 min) and spiked with 100 ng ml^{-1} internal standard (4.27 min).

above. Since lipophilic components, internal standard and navelbine tend to be very strongly retained on non-polar stationary phases, it was decided to reduce this problem by using a high percentage of acetonitrile or methanol in the mobile phase. Using 70% methanol or 45% acetonitrile in the mobile phase led to correct retention times. However, use of 70% methanol reduced the conductivity of the mobile phase and hence the sensitivity. On the other hand, we observed a rapid decrease in the sensitivity of the working electrode when using 45% acetonitrile. Although this problem was not studied in detail, we thought that a high percentage of acetonitrile in mobile phase could result in polymerization and consequent electrode neutralization. Nevertheless, it was necessary to obtain a compromise between a retention time of less than 10 min and good sensitivity and stability of the amperometric system. The final choice of organic modifier was 50% methanol with 8% acetonitrile, which enabled us to separate navelbine and the internal standard with a resolution of 2.80.

Different internal standards were investigated among the vinca alkaloids: vindesine, vincristine, vinblastine and 12-bromonavelbine. This last compound was kindly supplied by Pierre Fabre Labs. and eluted as expected just behind the navelbine peak. Unfortunately, however, it was not pure, and its chromatographic profile showed a discrete peak that eluted with the same retention time as navelbine. Modification of the mobile phase by varying the alkyl chain-length or the concentration of the counter-ion did not resolve this problem, so we decided not to use this compound. Whatever the mobile phase, vindesine, vincristine and vinblastine all elute before navelbine. Vindesine and vincristine elute near the solvent front, so vinblastine was chosen as internal standard because it elutes following the bulk of the electroactive constituents of plasma.

Detection procedure

From our preliminary investigations, it was concluded that UV detection was not suitable: many interferences arose when navelbine was determined in biological specimens. This could be avoided by using fluorescence detection, which has been described as a very selective and sensitive method for detecting vinca alkaloids [11,12]. Unfortunately, it was shown that both the optimum excitation and emission wavelengths were different for the internal standard and navelbine in the mobile phase used. So, although fluorescence detection is always easier to handle than electrochemical detection, this latter method was chosen not only because of its higher selectivity than that of UV detection, but also because it is a sensitive method for the determination of such compounds [13–15]. Following Smyth [13], glassy carbon was chosen as the material of the working electrode. The maximum signal-to-noise ratio for navelbine was achieved at a detection potential of 0.93 V. In order to shorten the stabilization period [14], the potential was adjusted to +1.00 V for 1 h and then decreased to 0.93 V every time a new mobile phase was used.

Linearity, limit of detection and selectivity

Linear standard curves were obtained by plotting peak-height ratios of navelbine and internal standard versus plasma concentrations (1, 5, 50, 200, 500 and 1000 ng ml^{-1}) or versus navelbine urinary concentrations (20, 50, 100, 250, 500 and 1000 ng ml^{-1}). Each value was the mean of eight measurements. The calibration curves for navelbine in plasma and urine, respectively, are described by the following equations: $y=0.005x+0.016$ ($r^2=0.999$) and $y=0.002x+0.058$ ($r^2=0.998$). The detection limit of the method was ca. 1 ng ml^{-1} in plasma and 20 ng ml^{-1} in urine, with in each case a signal-to-noise ratio greater than 3:1. Several pre-dose plasma samples from different subjects were tested for the absence of interfering endogenous compounds; no endogenous chromatographic interference was found at the retention time of either navelbine or the internal standard. Possible interferences due to co-administered drugs were evaluated and the results are given in Table I. No interference was noted with the drugs tested, although the metabolites of these compounds were not tested. On the other hand, although morphine eluted with a capacity factor less than that of vinblastine, a large solvent front interfering with the latter was observed in urine samples of patients who received the former.

This method could also be used for determination of vinblastine, using navelbine as internal standard.

TABLE I

SELECTIVITY OF THE HPLC SYSTEM

k' = capacity factor; N.D. = not detected; 100 μl of 1 mg ml^{-1} aqueous solution of each drug were injected. The k' value of vinblastine is 2.70 and that of navelbine is 5.20.

Drug	k'	Interferences	Drug	k'	Interferences
Acetylcysteine	N D.	–	Diprophylline	N.D.	–
Acetylsalicylic acid	N.D.	–	Doxycycline	0.14	–
Alizapride	N.D.	–	Floctafenine	N.D.	–
Aminophylline	1.96	–	Glafenine	0.54	–
Amiodarone	7.00	–	Indometacin	0 90	–
Amitriptylline	N.D.	–	Loperamide	N.D.	–
Amoxicilline	N.D.	–	Lorazepam	N.D.	–
Bromazepam	N.D.	–	Methylprednisolone	N.D.	–
Bromhexine	N.D.	–	Metoclopramide	N.D.	–
Caffeine	N.D.	–	Morphine	0.18	–
Carbocysteine	0.91	–	Noramidopyrine	0.45	–
Clavulanic acid	N.D.	–	Paracetamol	0.22	–
Clomipramine	N.D.	–	Prednisolone	N.D.	–
Clorazepate	N.D.	–	Ranitidine	N.D.	–
Dextropropoxyphene	1.10	–	Salbutamol	N.D.	–
Diazepam	N.D.	–	Theophylline	N.D.	–
Diclofenac	11.90	–			

Reproducibility and recovery

Within-day reproducibility was determined by carrying out eight determinations at concentrations of navelbine in the range 1–1000 ng ml^{-1} in plasma and 50–1000 ng ml^{-1} in urine. Day-to-day reproducibility was obtained from plasma spiked with 10, 20, 50, 100 and 500 ng ml^{-1} navelbine by carrying out eight determinations over fifteen days and nor more than one assay per day for each concentration. The data presented in Table II indicate that within-day and day-to-day coefficients of variation (C.V.) in plasma were less than 9%, except for the concentration near the limit of detection: in these cases (5 and 1 ng ml^{-1}), the C.V. were, respectively, ca. 11% and 20%. Within-day C.V. in urine were less than 13%.

Absolute recovery was studied by adding known amounts of navelbine to drug-free plasma at concentrations of 10, 100 and 1000 ng ml^{-1}. Eight extractions were performed for each concentration. Recoveries for these three concentrations were 75.4 ± 5.1, 75.4 ± 4.1 and 73.7 ± 4.1%, respectively. These results illustrate the consistency of the recoveries over the concentration range of interest.

Preliminary pharmacokinetic study

Using this method, we investigated the plasma kinetics of navelbine in eight patients, aged 51 to 74 years. The purpose of the study was explained to the patients and their informed consent was obtained orally. The protocol was approved by the local ethical committee. The patients were treated with na-

TABLE II

WITHIN-DAY AND DAY-TO-DAY REPRODUCIBILITIES

Added concentration (ng ml^{-1})	Within-day ($n=8$)				Day-to-day ($n=8$)	
	Plasma concentration measured (mean ± S.D.) (ng ml^{-1})	C.V. (%)	Urine concentration measured (mean ± S.D.) (ng ml^{-1})	C.V. (%)	Plasma concentration measured (mean ± S.D.) (ng ml^{-1})	C.V. (%)
1	1.10 ± 0.22	20.2	–	–	–	–
5	5.06 ± 0.54	10.7	–	–	–	–
10	10.00 ± 0.68	6.8	–	–	9.86 ± 0.86	8 7
20	–	–	–	–	19 83 ± 0.94	4.7
50	50.85 ± 3 20	6 3	40.59 ± 4.47	11.0	51.42 ± 3.20	6.2
100	101.14 ± 5.57	5.5	97.64 ± 12.64	12.9	98.58 ± 3.20	3.2
200	200.00 ± 5.30	2.6	–	–	–	–
250	–	–	287.82 ± 11.53	4.1	–	–
500	500.85 ± 13.69	2 7	505.29 ± 12.12	2.4	500.00 ± 17.31	3.5
1000	971.59 ± 53.99	5.6	988.82 ± 52.88	5.3	–	–

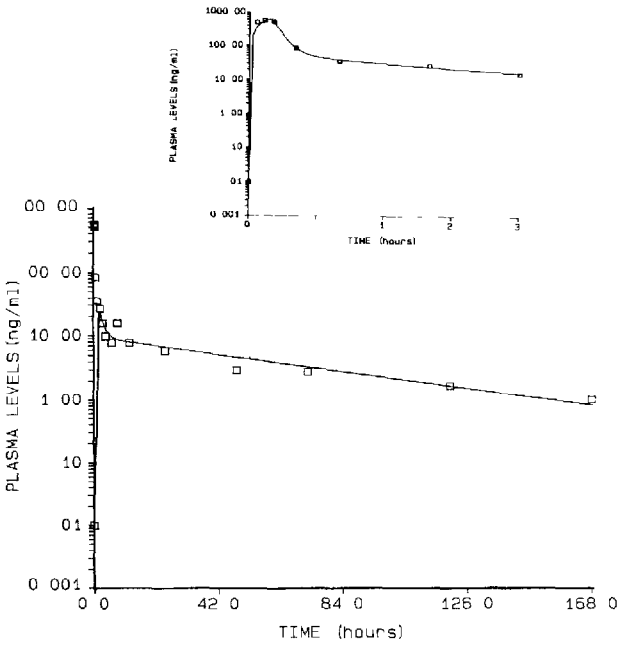

Fig. 2. Plasma concentration–time curve of navelbine, following 15-min infusion of 30 mg m^{-2} in one patient.

velbine for histologically documented neoplastic solid tumours. Pharmacokinetic parameters were calculated during the first course of navelbine therapy after doses of 30 mg m^{-2} were given as an infusion for 15 min. Plasma concentration values were fitted using the PHARM program of Gomeni [16]. A three-compartment model was employed consistent with the triphasic plasma concentration–time curves. Fig. 2 shows a representative log plasma concentration–time profile of navelbine in one patient. The C_{max} values ranged from 564 to 1234 ng ml^{-1}; the mean area under the curve from zero to infinity, the volume of distribution and clearance were 1013 ng ml^{-1} h, 64.1 l kg^{-1} and 0.92 l kg^{-1} h^{-1}, respectively. Large inter-individual variations were noted in the terminal half-life (mean $\pm$S.D. 46.7 $\pm$ 23.6 h). The method was also tested by determining the amount of navelbine in urine. Approximately 13.9% (3.1–28.6%) of the total dose administered was excreted unchanged after 168 h.

In conclusion, this HPLC technique is relatively easy to perform and appears to be reliable in determining navelbine in plasma at the nanogram level and in urine. It has been applied successfully to a study of the kinetic behaviour of navelbine, results of which are reported following a first administration. More complete pharmacokinetic investigations are in process.

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