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Short communication

Determination of vinorelbine in rabbit plasma by high-performance liquid chromatography with coulometric detection

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Abstract

A high-performance liquid chromatographic method was developed for the determination in plasma (400-μl sample) of a vinca alkaloid, vinorelbine. The analysis was performed by using an octadecylsilane column and heptanesulfonic acid as ion-pairing agent. This method used a new internal standard, teniposide, that permitted a good compromise between sensitivity and retention times (10.6 and 15.5 min for teniposide and vinorelbine, respectively). After a liquid–liquid extraction with diethyl ether, the extracts were injected into a reversed-phase system. The extraction efficiency was approximately 80% for both vinorelbine and the internal standard. The mobile phase was phosphate buffer (pH 3)–acetonitrile–methanol (50:30:20, v/v/v). Using coulometric detection, the limit of detection in plasma (400 μl) was 1 ng/ml. The intra-assay coefficients of variation were 10.95, 3.80 and 5.71% for 5, 500 and 1000 ng/ml, respectively, and the inter-assay coefficients of variation were 20.14, 14.27 and 10.67% for 5, 500 and 1000 ng/ml, respectively. A linear response was observed for the plasma calibration graph in the ranges 2.5–50 and 50–1000 ng/ml. This method was used to follow the time course of the concentration of vinorelbine in rabbit plasma after a single intravenous dose of vinorelbine (30 mg/m^2) and seems to be suitable for studying the pharmacokinetics of vinorelbine in rabbit.

1. Introduction

Vinorelbine is a relatively new anti-cancer agent. Although still under clinical development, it has generated a relatively large amount of experimental and clinical data [1]. Among its advantages, vinorelbine has a wide range of anticancer activity and possesses little if any extrahaematological toxicity [2]. Its efficacy against non-small-cell lung cancer and breast cancer has recently been demonstrated [3].

To date, few methods for determining vinorelbine levels in biological fluids have been published. The methods known include a radioimmunoassay technique used to determine its pharmacokinetics [4] and several high-performance liquid chromatographic (HPLC) techniques [5–7]. Among the different detection methods used, UV detection is neither very selective nor sensitive, and hence is not suitable for the determination of vinorelbine in biological fluids [6,7]. Fluorescence [8] and electrochemical detection may offer potential advantages in terms of selectivity and sensitivity. Nicot et al. [9] investigated the use of an electrochemical

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detector in the amperometric mode, but the coulometric mode has not previously been evaluated.

This paper describes a simple and sensitive HPLC method for the determination of vinorelbine in rabbit plasma, suitable for pharmacokinetic investigations, using a reduced volume of plasma, coulometric detection and an internal standard.

2. Experimental

2.1. Chemicals and reagents

Vinorelbine was obtained from Pierre Fabre Médicament (Paris, France), and teniposide, the internal standard, was kindly provided by Sandoz (Rueil-Malmaison, France). HPLC-grade acetonitrile, methanol and diethyl ether were purchased from Solvants Documentations Synthèses (Peypin, France). Sodium dihydrogenphosphate (Sigma, Paris, France), heptanesulfonic acid (Interchim, Montluçon, France) and all other reagents were of analytical-reagent grade and were used as supplied.

2.2. Chromatography

Chromatographic analyses were performed using an HPLC system consisting of an Applied Biosystems (Roissy, France) Model 400 A solvent delivery system connected to a Coulochem 5100 A coulometric detector (Environmental Sciences, Bedford, MA, USA). The chromatograms were recorded and peak areas integrated on a Data Jet Model SP4600 integrator (Spectra-Physics, Fremont, USA).

Chromatographic separations were performed on an octadecylsilane column (LiChrospher 100 RP-18, 250 × 4 mm I.D., 5 μm) (Merck, Darmstadt, Germany). The mobile phase was sodium dihydrogenphosphate buffer (20 g/l)–acetonitrile–methanol (50:30:20, v/v/v). Heptanesulfonic acid (0.8 g/l) was added to the aqueous sodium dihydrogenphosphate solution and the pH was adjusted to 3.0 with orthophosphoric acid to improve the peak shape. The mobile phase was filtered through a 0.4-μm filter and degassed before use. The flow-rate was 1 ml/min.

Electrochemical detection was performed with a potential of +0.40 V for the first cell (clean-up cell) and +0.70 V for the second cell (detection cell). A guard cell with a potential of +0.90 V was placed before the injector to oxidize the electroactive impurities of the recycled mobile phase.

2.3. Extraction procedure

A 20-μl volume of internal standard solution (10 μg/ml teniposide) and 1600 μl of diethyl ether were added to 400 μl of plasma. The mixture was shaken on a vortex mixer and centrifuged for 2 min at 1000 *g* at room temperature. The organic layer (supernatant) was transferred into a clean tube and evaporated to dryness under a stream of air at 30°C. The dried residue was then dissolved in 200 μl of the mobile phase and 100 μl were injected into the chromatographic system.

2.4. Quantitative analysis

The ratio of the detector response (peak area) for vinorelbine to that for the internal standard was determined for known concentrations of vinorelbine in plasma. Two calibration graphs ranging from 2.5 to 50 ng/ml ($n = 4$) and from 50 to 1000 ng/ml ($n = 5$) were prepared in duplicate by spiking plasma with increasing amounts of vinorelbine to determine the concentration of unknown samples.

The intra-assay precision was determined by analysing ($n = 5$) plasma samples spiked with vinorelbine at concentrations of 5, 500 and 1000 ng/ml and comparing the ratio of the area under the curve for vinorelbine to that for the internal standard. The inter-assay precision was evaluated by analysing plasma samples (5, 500 and 1000 ng/ml) on four different days.

3. Results and discussion

3.1. Extraction procedure

In previous studies [6,9,10], diethyl ether was used for vinorelbine extraction. Several organic solvents were evaluated in order to improve the overall recovery of known liquid–liquid extraction procedures. Among these, dichloromethane was not able to extract vinorelbine, and emulsions were obtained with chloroform, chloroform–methanol (80:20) and dichloromethane–methanol (80:20). Diethyl ether proved to be the best solvent. Dissolution of the dry residue in the mobile phase, followed by washing with hexane, as done by Nicot et al. [9], did not improve the selectivity of the method.

Another approach, consisting of deproteination by addition of acetonitrile, perchloric acid, methanol or zinc sulfate–NaOH [11], followed by direct injection into the chromatographic system, yielded unsatisfactory chromatograms containing many peaks of unknown origin. Further purification by extraction of the supernatant with diethyl ether did not improve this approach.

Consequently, we decided to use a simple liquid–liquid extraction with diethyl ether. The extraction recovery from plasma samples spiked with vinorelbine at concentrations of 50 and 500 ng/ml were 79 ± 10% and 80 ± 4%, respectively ($n = 5$).

3.2. Chromatographic separation

Owing to the pK_a value of vinorelbine (5.4), this drug is protonated when the pH of the mobile phase is adjusted to 3.0. Hence it can form an ion pair in the presence of an anionic counter ion such as heptanesulfonate, and can be separated on non-polar stationary phases.

The choice of the internal standard presented many difficulties. Several vinca alkaloids commonly used, such as vindesine, vincristine and especially vinblastine [6,8–10], were evaluated without success owing to their short retention times. Modification of the mobile phase did not resolve this problem as they still eluted either too early (with the solvent front) or with endogenous peaks. Teniposide was chosen as the internal standard for several reasons. First, it is well resolved from endogenous peaks and from vinorelbine. Second, its retention time, in comparison with that of vinorelbine, remains only slightly changed on varying the proportion of methanol in the mobile phase.

In order to optimize the efficiency and selectivity of the chromatographic separation, the effect of varying the proportions of acetonitrile and methanol in the mobile phase was investigated with a fixed phosphate buffer proportion of 50%. A high proportion of acetonitrile tended to shorten the retention time of vinorelbine whereas a high proportion of methanol lengthened it. The final choice was a compromise between sensitivity and resolution.

Under the conditions described, vinorelbine and teniposide gave sharp and well separated peaks with retention times of 15.5 and 10.6 min, respectively. Typical chromatograms obtained after extraction from plasma are shown in Fig. 1.

3.3. Determination of optimum detection conditions

The detection conditions were optimized to find the maximum signal-to-noise ratio for vinorelbine. Accordingly, peak areas were measured after repeated injections of a vinorelbine solution (1000 ng/ml) and application of voltages ranging between +0.4 and +0.95 V to the detection cell. The hydrodynamic voltammograms obtained are shown in Fig. 2. The maximum of these plots corresponds to the optimum potential to be applied for the detection of vinorelbine (+0.70 V). Also, as can be seen on these plots, little if any vinorelbine is oxidized at a potential of +0.40 V, so this potential was chosen for the clean-up cell. The limit of detection, based on a signal-to-noise ratio of 3, was 1 ng/ml in plasma.

3.4. Linearity of response and reproducibility

Calibration graphs were obtained by plotting the peak-area ratio of vinorelbine to the internal standard versus vinorelbine plasma concentra-

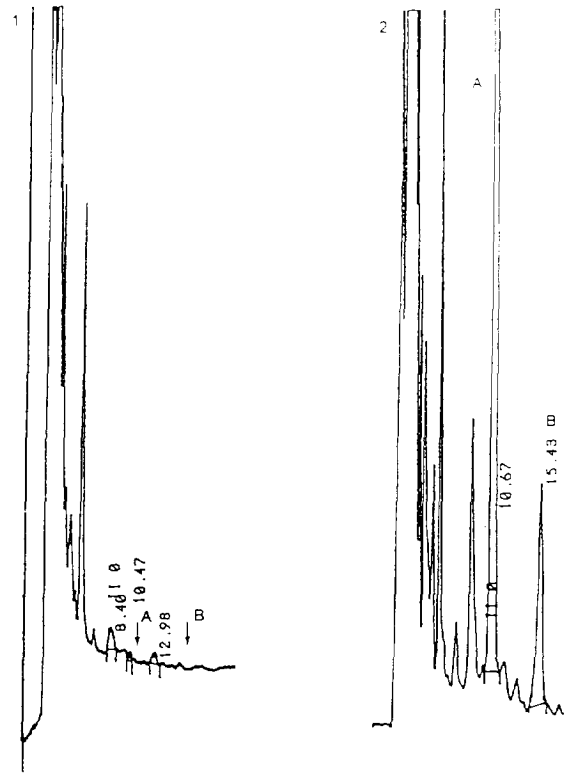

Fig. 1. Chromatograms of plasma extracts. (1) Blank plasma extract; (2) plasma extract containing (A) 500 ng/ml of internal standard and (B) 25 ng/ml of vinorelbine.

tion. However, as found by Van Tellingen et al. [10], two calibration graphs were necessary for the accurate determination of high and low vinorelbine concentrations. Accordingly, calibration graphs were set up for plasma concentrations ranging between 2.5 and 50.0 ng/ml and between 50 and 1000 ng/ml. They were linear in the investigated ranges and described by the following equations: $y = 0.0088x + 0.0389$ ($r = 0.979$) and $y = 0.0035x + 0.2612$ ($r = 0.997$) for the ranges 2.5–50 ng/ml and 50–1000 ng/ml, respectively, where y = peak-area ratio and x = concentration (ng/ml).

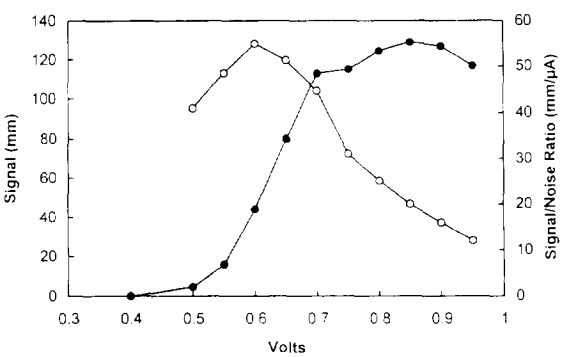

Fig. 2. Hydrovoltammogram for vinorelbine. ● = Signal as a function of potential; ○ = signal-to-noise ratio as a function of potential.

The reproducibility of the method was studied by repeated analysis of known concentrations. Table 1 shows that the within-run (intra-day) and between-run (inter-day) reproducibilities are good with intra-day coefficients of variation of about 10% at the lower plasma concentrations. The inter-day variability was more sensitive to the composition of the mobile phase, as reflected by higher coefficients of variation in the low concentration range.

3.5. Preliminary pharmacokinetic study

The performance of the assay was evaluated by determining plasma concentrations of vinorelbine following an intravenous bolus injection of a dose of 30 mg/m^2 into one rabbit. Blood samples (1 ml) were collected in eppendorf tubes containing lithium heparin at 0, 0.25, 0.5, 0.75, 2, 3, 4, 5, 6 and 7 h. The relationship between plasma concentrations of vinorelbine in this animal and time is presented in Fig. 3. At 0.25 h the vinorelbine concentration was 118.2 ng/ml, then it rapidly decreased and was only 22.8 ng/ml at 2 h. After this time, the profile of concentrations suggests that vinorelbine is subject to an enterohepatic cycle. Application of the method to the study of the pharmacokinetics of

Table 1
Intra-assay ($n = 5$) and inter-assay variability of the extraction procedure for plasma

Concentration (ng/ml)	Coefficient of variation (%)	
	Intra-assay ($n = 5$)	Inter-assay
5	10.95	20.14
500	3.80	14.27
1000	5.71	10.67

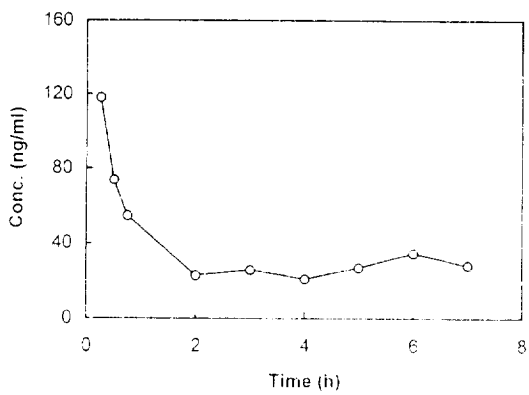

Fig. 3. Plasma concentration versus time plot for vinorelbine after an intravenous bolus injection of 30 mg/m^2.

vinorelbine in a rabbit brain neoplasm model is planned.

4. Conclusion

The described chromatographic method represents a simple alternative for the determination of vinorelbine in plasma. HPLC coupled with coulometric detection is used because of its high selectivity and sensitivity. This method requires only small volumes of plasma, and was applied to the study of vinorelbine pharmacokinetics in rabbit plasma over 48 h. The method was sufficiently sensitive to determine vinorelbine plasma levels and the detection limit of 1 ng/ml was suitable for the experiment.

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