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Short Communication

High-performance liquid chromatographic determination of navelbine in MO_4 mouse fibrosarcoma cells and biological fluids

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ABSTRACT

A high-performance liquid chromatographic method is described for separating and determining navelbine and possible metabolites in plasma, cell culture medium and MO_4 cells. Navelbine is extracted from these fluids by ion-pair extraction with sodium octylsulphate as the counter-ion at pH 3. The system uses a cyano column as the stationary phase and a mobile phase of acetonitrile–0.12 *M* phosphate buffer (pH 3) (60:40, v/v). Application of the method to a study of the pharmacokinetic behaviour of navelbine in MO_4 mouse fibrosarcoma cells is reported.

INTRODUCTION

Navelbine (Fig. 1) is a new semisynthetic vinca alkaloid, which chemically differs from vinblastine by substitution on the catharantine moiety of the molecule [1]. Preclinical studies revealed good antitumour activity against the murine leukemias L1210 and P388, and also against vinca alkaloid-resistant P388 strains [2]. It has shown to be active in non-small-cell lung cancer, breast cancer, Hodgkin's disease and other solid tumours [3]. The pharmacokinetics of navelbine have been determined by radioimmunoassay (RIA) [4,5] and by use of the tritiated drug [6]. There is a view that a significant proportion of the parent drug is biotransformed and not detected by RIA meth-

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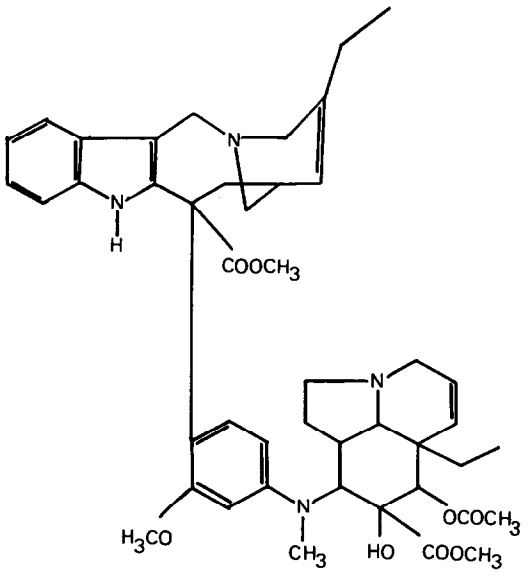

Fig. 1. Molecular structure of navelbine sulphate.

ods [6]. This lack of specificity could be resolved by a reliable high-performance liquid chromatographic (HPLC) method. Recently one group has been developing an HPLC method for navelbine determination in blood and urine [7,8]. This method proved to be very sensitive and selective, but the extraction recovery was somewhat low: 66.8–68.1%. In order to study the behaviour of navelbine in cellular uptake models i.c. MO_4 cells, we developed an HPLC method with UV detection to determine navelbine.

EXPERIMENTAL

Apparatus

A Varian 2010 HPLC pump and a Varian 2030 variable-wavelength UV detector, operated at 220 nm, and a Gilson-231 autoinjector (sample loop 100 μl) were used. The flow-rate was 1.0 ml/min. The chromatograms were recorded and integrated with a Hitachi D-2000 integrator. The stainless-steel column (250 × 4 mm I.D.) was packed with LiChrosorb CN, particle size 5 μm (Merck, Darmstadt, Germany). A guard column (30 × 4 mm I.D.) filled with the same material was used to protect the analytical column. The mobile phase was acetonitrile–0.12 *M* phosphate buffer (pH 3) (60:40, v/v).

All experiments were carried out at ambient temperature. All laboratory glassware was silanized with dichlorooctamethyltetrasiloxane (Surfasil®, Pierce, Rockford, IL, USA) diluted with acetone.

Standards and reagents

Navelbine sulphate and deacetylnavelbine sulphate were of analytical purity and kindly provided by Pierre Fabre Labs. (Paris, France). Vinblastine sulphate (Velbe®) and vincristine sulphate (Oncovin®) were of pharmaceutical purity and kindly provided by Eli Lilly (Brussels, Belgium). Chloroform, sodium octylsulphate, phosphoric acid, sodium dihydrogenphosphate monohydrate and potassium chloride were pro analysis quality. Acetonitrile and dichloromethane were liquid chromatographic grade. All these solvents were obtained from Merck. A phosphate buffer (pH 3, 0.4 *M*) containing $5 \cdot 10^{-2}$ *M* octylsulphate was used for the extraction. For the mobile phase a phosphate buffer (pH 3, 0.12 *M*) was used.

Cell cultures

MO_4 cells are viral-transformed fibrosarcoma-like C_3H mouse cells [7]. They are maintained in monolayer culture using Eagle's Minimum Essential Medium Modified (Modified MEM, Gibco Europe, Paisley, UK) supplemented with 10% foetal calf serum (Gibco Europe) and 0.05% L-glutamine (Difco Labs., Pasture, Brussels, Belgium). Penicillin G (100 E/ml) and streptomycin sulphate (100 μg/ml) were added to the culture medium. Hereafter the supplemented culture medium will be called MEM 10. The cells were placed in culture vessels of area 75 cm^2 (Nunc, Gibco Europe) and kept in a CO_2 incubator (Forma Scientific, Brugman TTO, Brussels, Belgium) at a temperature of 37°C.

Extraction and recovery of navelbine from plasma

A 4-ml volume of plasma was pipetted into a centrifuge tube equipped with a PTFE-covered screw-cap. Deproteinization was carried out by

dropwise addition of 8 ml of acetonitrile under continual vortexing. After centrifugation for 30 min, the supernatant was pipetted into another centrifuge tube, and the acetonitrile was evaporated in a water-bath at 60°C under a gentle stream of nitrogen. A 10-ml aliquot of a phosphate buffer (pH 3, 0.4 *M*) containing $5 \cdot 10^{-2}$ *M* sodium octylsulphate and 5 ml of chloroform was added and shaken for 30 min in a shaking bath. After centrifugation, 4 ml of the organic phase were put into a Reacti vial and evaporated to dryness at 30°C under a gentle stream of nitrogen. The residue was dissolved in 200 μl of dichloromethane, and 100 μl were injected into the HPLC system.

Quantitation was carried out by intrapolation on an extracted curve in plasma (peak-area ratio of drug to internal standard *versus* concentration of drug). Vincristine was used as the internal standard (I.S.) in this method. For the determination of the extraction recovery, quantitation was performed by comparison with a standard curve obtained from an aqueous solution.

Extraction and recovery of navelbine from MO_4 cells and MEM 10

The MO_4 cells were lysed before the extraction procedure was carried out. First the cells were scraped from the bottom of the culture vessel with a policeman, and single cells were obtained by means of a syringe. The cell suspension was then placed in a centrifuge tube and centrifuged for 15 min at 700 *g*. The supernatant was discarded and 4 ml of potassium chloride (0.075 *M*) were added to obtain cell lysis in a hypotonic medium at 37°C for 30 min. This solution was then put in an ultrasonic bath for 1 min to ensure complete cell lysis.

The extraction procedure was then performed, starting from 4 ml of MEM 10 or 4 ml of potassium chloride (containing the lysed cells) with the addition of 100 μg of vincristine as I.S. The recovery was calculated as described previously.

In each series of experiments, on the different days of sample collection, a supplementary culture vessel was treated in the same way except that the cells were not lysed but trypsinized for cell count. The number of non-vital cells was evaluated using the tryptan blue exclusion test. The number of dead cells was not taken into account in calculating the navelbine concentration in the cells.

Preparation of standard curves

Stock solutions of navelbine were prepared in concentrations of 1 μg/ml and 1 mg/ml. From these standards, solutions were used to spike plasma in the following final concentrations: 1.25 ng/ml, 2.5 ng/ml, 12.5 ng/ml and 125 ng/ml in a first series; 2 μg/ml, 4 μg/ml, 6 μg/ml and 8 μg/ml in a second series. MEM 10 was spiked in the following concentrations: 5 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml in a first series, and 2 μg/ml, 4 μg/ml, 6 μg/ml and 8 μg/ml in a second series. Stock solutions were stored at 4°C between uses.

Reproducibilities

Plasma and MEM 10 were spiked with 25, 50, 100 or 200 ng/ml. A standard curve was made with these concentrations for five consecutive days. Six samples were taken each day for each concentration.

Detection limit

The limit of detection was defined as the lowest concentration of navelbine in aqueous solution and in MEM 10, corresponding to a signal-to-noise ratio of 3.

Selectivity of the system

Interference studies were carried out with vinblastine, vincristine, vindesine, deacetylnavelbine (a possible metabolite) and 31 other substances, commonly consumed by cancer patients. These drugs were the same as already tested in a previous study [8].

Cellular uptake of navelbine (preliminary results)

Navelbine was added to MEM 10 in different concentrations: 0.01, 0.1 and 1 μg/ml MEM 10. Vinblastine uptake at 0.1 μg/ml MEM 10 by the MO_4 cells was used as the control uptake curve. Vincristine was used as the I.S. Samples of MEM

10 and cells were taken at times 0.5, 6, 12, 24 and 48 h. At least six samples for every point of the curve were assayed. The intracellular concentration, expressed as μg/ml cell volume, was calculated based on the concentration of the drug (expressed in μg/ml potassium chloride solution), the number of vital cells, and the mean volume of one MO_4 cell (*ca.* 10^{-6} μl, calculation based on a value of 12.5 μm for the mean diameter of an MO_4 cell). The accumulation ratio (AccR) is the ratio of the measured intracellular concentration to the measured extracellular concentration of the drug, *i.e.* in MEM 10.

RESULTS AND DISCUSSION

Chromatography, selectivity and detection limit

Fig. 2 gives an example of a chromatogram of (A) an extract of a blank sample of plasma and (B) an extract of plasma spiked with 10 ng/ml navelbine (peak N) and 10 ng/ml vincristine (peak V). Fig. 3 shows a chromatogram of (A) an extract of a blank sample of MEM 10, (B) an extract of MEM 10 solution containing 200 ng/ml navelbine (peak N) and 200 ng/ml vincristine (peak V) and (C) an extract of MO_4 cells containing 200 ng/ml navelbine. The cell suspension was spiked with 200 ng/ml vincristine. Fig. 4 shows a chromatogram of an extract of MEM 10 spiked with 20 ng/ml vincristine (peak V), 5 ng/ml deacetyl navelbine and 12.5 ng/ml navelbine. These two components can easily be distinguished from each other. Therefore the described HPLC method seems to be suitable for the separation and determination of navelbine and its possible metabolites in biological fluids.

The detection limit of navelbine in aqueous solution is 2.5 ng/ml. The detection limit of spiked plasma or MEM 10, after extraction starting from 4 ml of solution, is 1.25 ng/ml. The technique of Jehl *et al.* [8] could determine navelbine at a level of 0.5 ng/ml, which is in the same range as our method.

Extraction and recovery of navelbine from plasma and MEM 10

The extraction procedure is based on an ion-pair extraction at pH 3 with octylsulphate as counter-ion. The same procedure has already been successfully applied for basic drugs [9] and

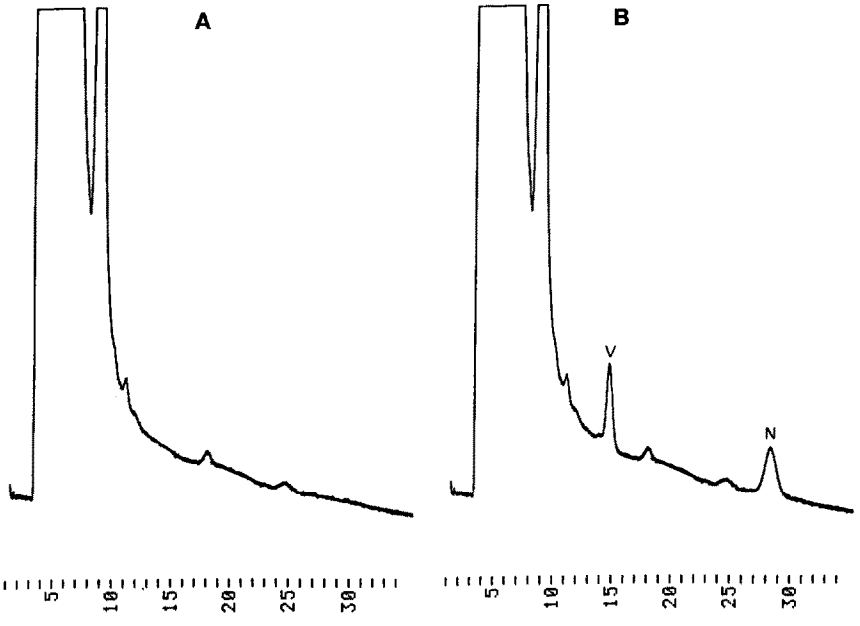

Fig. 2. HPLC of an extract of (A) a blank sample of plasma and (B) a sample of plasma spiked with 10 ng of navelbine (peak N) and 10 ng of vincristine (peak V) as internal standard. Time scales in min.

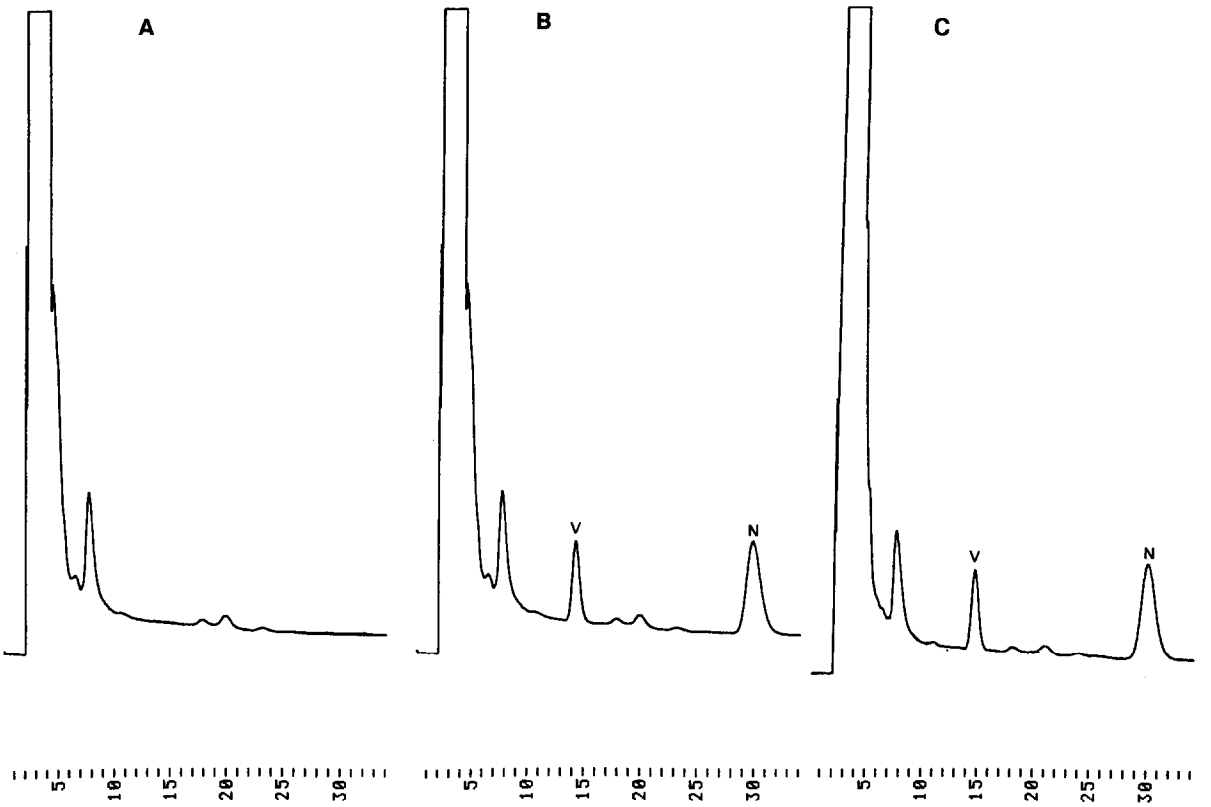

Fig. 3. HPLC of an extract of (A) a blank sample of MEM 10, (B) a sample of MEM 10 spiked with 200 ng of navelbine (peak N) and 200 ng/ml vincristine (peak V) as internal standard, and (C) a sample of lysed MO_4 cells containing 200 ng of navelbine (peak N) and 200 ng/ml vincristine as internal standard (peak V). Time scales in min.

vinca alkaloids [8] in biological material. A variant of the technique has been developed for culture medium and cell suspensions [10]. For navelbine we had to adapt the ionic strength of the buffer of the mobile phase (0.12 *M* instead of 0.08 *M*), resulting in a slower elution of the compounds, but we were able to retain the initial ionic strength of the extraction buffer (0.4 *M*).

The extraction recovery was 93 ± 5.81% for 7.14 ng/ml, 100 ± 6.74% for 100 ng/ml and 110 ± 3.19% for 8 μg/ml plasma. The overestimation of the recovery at very high concentrations is probably due to the inertia of the automatic peak-area integration system.

The extraction recovery from MEM 10 was 95% for 6.55 ng/ml, 101 ± 4.59% for 100 ng/ml and 99 ± 3.13% for 8 μg/ml. Previous extraction methods [7] yielded lower recoveries (66.8–68.1%), but combined with a sensitive HPLC system can provide adequate detection limits (0.5 ng/ml). These results confirm that the extraction procedure and HPLC method are suitable for the determination of navelbine and other vinca alkaloids in biological fluids as well as in cell culture systems.

Standard curves

The linearity of the standard curves was checked for navelbine concentrations in aqueous solution, in plasma and in MEM 10. The standard curve for extraction from aqueous solution can be described as $y = ax + b$, where $a = 0.0110$, $b = 0.0457$ and the correlation coefficient (r) = 0.9982. For plasma the curve parameters are $a = 0.0109$, $b = 0.0535$, and $r = 0.9931$. The values for MEM 10 at lower concentrations (5–

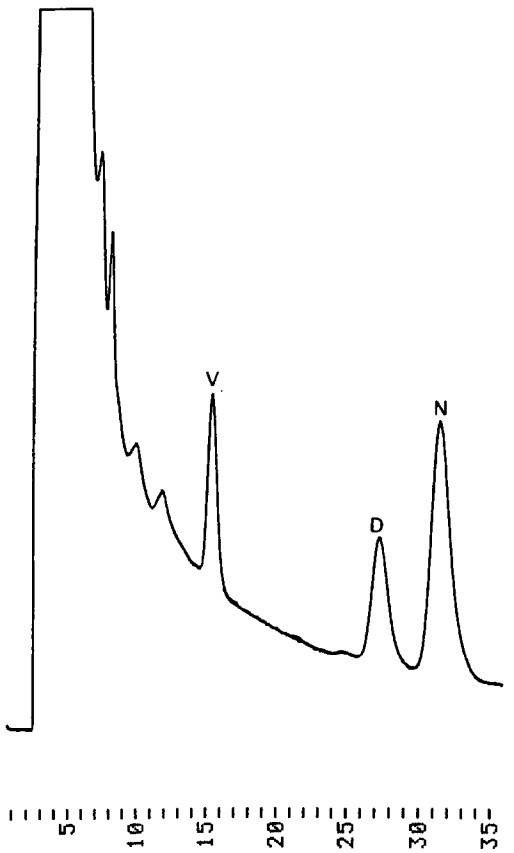

Fig. 4. HPLC of an extract of MEM 10 spiked with 20 ng/ml vincristine (peak V), 5 ng/ml deacetylnavelbine (peak D) and 12.5 ng/ml navelbine (peak N). Time scale in min.

200 ng/ml) are $a = 0.0203$, $b = 0.0106$ and $r = 0.9998$. At higher concentrations (2 μg/ml to 8 μg/ml MEM 10) the values are $a = 0.06756$, $b = 0.020$, and $r = 0.9982$.

These results confirm the reliability of the method, and show that navelbine may be determined in a wide range of concentrations, from 5 ng/ml to 8 μg/ml, especially in culture media.

Between-day reproducibility

The results of the between-day variation of extraction recovery of navelbine from plasma and MEM 10 are listed in Table I. The coefficients of variation of the experiments with 100 ng/ml navelbine spiked solutions are within acceptable limits.

These results obtained for lower concentrations (10 and 50 ng) are similar, and they are less than 5% for concentrations from 500 ng/ml to 8 μg/ml.

TABLE I

BETWEEN-DAY REPRODUCIBILITY OF EXTRACTION OF NAVELBINE (100 ng/ml) FROM PLASMA OR MEM 10

Day	Plasma		MEM 10	
	Recovery (%)	C.V. (±%)	Recovery (%)	C.V. (±%)
1	100	4.04	104	3.63
2	104	11.9	101	2.68
3	94	7.39	104	6.55
4	93	7.04	95	6.94
5	109	8.23	102	6.11

Cellular accumulation of navelbine and vinblastine by MO_4 cells

The results of these preliminary uptake studies are presented in Fig. 4. In previous studies we observed that vinblastine accumulation is a dose-related phenomenon, with an early accumulation mechanism and a second dose-induced accumulation period [10,11]. The results for navelbine presented here indicate that this semi-synthetic vinblastine derivative exhibits the same profile in MO_4 cellular uptake as its parent compound. More extensive studies are needed to confirm this, but nevertheless the method described here is suitable for these experiments.

CONCLUSION

We developed a method permitting the determination of navelbine in biological fluids, in MO_4 mouse fibrosarcoma cells, and in the corresponding culture medium. Other vinca alkaloids, vinblastine, vincristine or vindesine, can be used as an I.S. or as a control. The method involves ion-pair extraction and HPLC–UV for the quantitative determination. The procedure seems to be suitable for further elucidation of the cellular and clinical pharmacokinetics of navelbine.

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