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Short Communication

Bio-analysis of vinorelbine by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A simple and selective procedure for the determination of vinorelbine, a new semi-synthetic vinca alkaloid, is presented. The method is based on ion-exchange high-performance liquid chromatography on normal-phase silica with fluorescence detection, combined with liquid–liquid extraction using diethyl ether for sample clean-up. The absence of endogenous interferences and the excellent chromatographic behaviour of vinca alkaloids provides accurate results even at low concentrations. The limit of determination in plasma is 1.5 μg/l (500-μl sample). Reproducible recoveries in urine were obtained if 10–50 μl of sample were processed supplemented with 500 μl of blank plasma.

INTRODUCTION

Vinorelbine, nor-5′-anhydrovinblastine (navelbine, NVB) (Fig. 1), is a new semi-synthetic vinca alkaloid, which has shown promising anti-tumour activities against several human malignancies [1,2]. Furthermore, this compound compares favourably with other vinca alkaloids [vincristine, vinblastine (VBL)] with respect to neurotoxicity [1]. These results have increased the interest in the pharmacokinetic properties of this compound in comparison to other vinca alkaloids.

Most pharmacokinetic studies of NVB presented to date have been performed by radioimmunoassay (RIA) or by the administration of radiolabelled drugs [3–6]. However, the specificity

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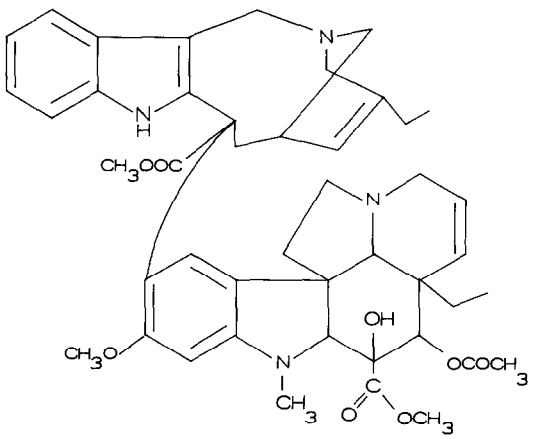

Fig. 1. Stucture of NVB.

of these methods for the parent drug may be insufficient owing to the presence of structurally related compounds (metabolites), and the results from studies using these methods should be interpreted with caution. Recently, two papers have been published dealing with the bio-analysis of NVB by high-performance liquid chromatography (HPLC), based on ion-pair reversed-phase chromatography with electrochemical detection [7] or reversed-phase chromatography with UV detection [8]. The problems that we encountered with reversed-phase liquid chromatography during our work on the bio-analysis of experimental vinca alkaloids [9] led us to develop a chromatographic method based on ion-exchange (IE) HPLC for this type of compound [10,11]. IE-HPLC also appears to be an excellent analytical tool for the measurement of NVB in biological samples. This paper describes an alternative analytical procedure for NVB, based on IE-HPLC with fluorescence detection, which will be used in a comparative pharmacokinetic study of NVB and several other vinca alkaloids.

EXPERIMENTAL

Materials and reagents

VBL and desacetylvinblastine (DVBL) were obtained from the Medgenix Group (Fleurus, Belgium). NVB originated from Centre de Recherche Pierre Fabre (Paris, France). All other reagents were purchased from E. Merck (Darmstadt, Germany) and were of analytical quality, except for diethyl ether and acetonitrile, which were of HPLC grade. Blank human plasma was obtained from healthy donors. Water purified by the Millipore-Q system (Waters, Bedford, MA, USA) was used throughout.

Sample preparation

Volumes of 500 μl of plasma and 50 μl of internal standard (I.S.) solution (1 mg/l DVBL in acetonitrile) and 4 ml of diethyl ether were pipetted into a glass tube equipped with a PTFE-covered screw cap. The tubes were shaken vigorously for 10 min, followed by centrifugation for 10 min at 1000 *g* (4°C). The aqueous phase was frozen at −20°C for 60 min. The organic phase was decanted into a clean glass tube and evaporated to dryness under a stream of nitrogen (37°C). The residue was dissolved in 100 μl of acetonitrile by sonication for 5 min. An aliquot of 80 μl was subjected to chromatography.

The extraction procedure for urine was identical, except that 10–50 μl urine were extracted together with 500 μl of blank plasma.

High-performance liquid chromatography

Chromatographic analyses were performed using an HPLC system consisting of a Spectroflow SF400 pump, a Spectroflow 980 fluorescence detector (Kratos, Ramsey, NJ, USA) and a Model 360 autosampler (Kontron, Basel, Switzerland). Samples were chromatographed on a stainless-steel column (250 mm × 2 mm I.D.) packed with 5-μm Spherisorb Si (Phase Separations, Queensferry, UK). The mobile phase was acetonitrile–10 m*M* trisodium citrate buffer (adjusted to pH 3.0 with hydrochloric acid) (85:15, v/v) and contained 10 m*M* tetrabutylammonium bromide (TBABr). The column was eluted at ambient temperature at a flow-rate of 0.2 ml/min. The excitation monochromator was set at 270 nm, and emission was monitored using a 320-nm long-pass filter. Integration was done using a WINNER-4 data station (Spectra Physics, San Jose, CA, USA). Ratios of peak areas of NVB and the I.S. were used for quantitative calculations.

Calibration

Standard samples were prepared by diluting the NVB stock solutions (10 mg/ml) in blank hu-

man plasma to concentrations of 50 and 1000 μg/l, and were stored at −20°C. From these standard samples a calibration curve was constructed in plasma (concentrations 5, 10, 20, 50, 100, 200, 400 and 1000 μg/l) just prior to analysis.

The calibration curves were calculated by unweighted least-squares linear regression analysis. Two calibration curves (ranges 5–50 and 50–1000 μg/l) were computed for accurate quantification.

Blank plasma spiked with NVB (low and medium concentrations) and two urine specimens from patients served as control samples.

Validation

The accuracy of the method at each standard concentration was judged from the percentage relative concentration residuals (%RCR), which are defined as %RCR = 100 (RC − NC)/NC, where RC and NC represent the interpolated and nominal concentration, respectively [12].

The within-run relative standard deviations (R.S.D.) in plasma were calculated from the compound variance of the calibration standards assayed in duplicate with each series. In urine, the within-run R.S.D. was determined from the compound variance of the control samples assayed in quadruplicate with each series. Run-to-run R.S.D. were calculated from the control samples.

RESULTS AND DISCUSSION

Chromatography

During the past few years several analytical methods for vinca alkaloids based on HPLC have been presented [7–11,13,14]. In many cases reversed-phase chromatography in combination with oxidative electrochemical detection was used [7,9,13]. IE-HPLC combined with fluorescence detection appears to be an attractive alternative [10,11,14], featuring good chromatographic behaviour, column-to-column reproducibility, and the capability of separating a variety of vinca alkaloids and metabolites under uniform chromatographic conditions. The native fluorescence properties of vinca alkaloids, which are enhanced by high proportions of organic solvent in the mobile phase, yield better sensitivities with a potentially higher selectivity compared with UV detection, whereas compared with electrochemical detection, fluorescence detection is equally sensitive but much more robust.

Sample preparation

In previous papers, the use of liquid–liquid extraction with chloroform as a procedure for the pretreatment of samples in the analysis of several vinca alkaloids in biological samples has been described extensively. In the case of NVB, extraction with chloroform yielded only low recoveries. We therefore adopted liquid–liquid extraction with diethyl ether, as used by others [5,6]. Although the use of diethyl ether was convenient, we encountered some problems that have not been reported before. The extraction recovery of NVB at low concentrations (<50 μg/l) was found to be lower than with higher concentrations of the drug, which resulted in a non-linear calibration curve (Table I). In such cases some investigators prefer the use of non-linear curve-fitting [12], but for NVB acceptable results were obtained if the calibration curve was devided into two ranges: 0–50 and 50–1000 μg/l.

Another problem was the variation in the absolute recovery of NVB from different urine samples if they were processed as described before [5,6]. These investigators extracted a 1-ml sample supplemented with phosphate buffer (pH 7.0) for pH control. Under these conditions we found the absolute recovery of NVB (100 μg/l) to be 49.2 ± 23.1% (mean ± S.D.) (n = 11). With the use of VBL as the I.S., this effect was only partly compensated as the ratio NVB/VBL was found to be 0.707 ± 0.091 (R.S.D. = 12.9%) (n = 11), and with DVBL as the I.S. the results were even worse. Better results were obtained when the sample size was limited (up to 50 μl) with the addition of 500 μl of blank plasma. The recovery of NVB was then found to be 67.8 ± 4.9% (n = 11), with both DVBL and VBL being equally suitable as the I.S. [R.S.D. for the ratios NVB/DVBL and NVB/VBL were 4.9% and 4.1%, respectively (n = 11)]. An advantage is that under these conditions the recoveries of NVB and the I.S. from plasma and urine are equal, and all samples can be quantitated using the same calibration curve constructed in plasma. DVBL was selected as the I.S., as it is well separated from NVB and a metabolite, probably desacetylnavel-

TABLE I

REPRODUCIBILITY OF THE ASSAY

Concentration (μg/l)	%RCR[a]	%RCR[b]	Within-run R.S.D. (%)	Run-to-run R.S.D. (%)
5	−22.5	−9.8	10.7	
10	−13.2	−3.3	8.4	
20	−7.6	−0.4	6.4	
50	−1.6	0.9	4.4	
100	−1.5		4.6	
200	−4.2		2.6	
400	0.2		4.0	
1000	2.6		5.7	
18[c]			7.9	10.1
146[c]			4.8	6.1
8850[d]			2.4	3.7
1430[e]			2.2	3.6

[a] These %RCR were obtained if unweighted least-squares linear regression analysis was used to fit a calibration curve through the whole concentration range, and clearly demonstrate the non-linearity in the low concentration range.

[b] These %RCR resulted when the calibration curve was divided into two ranges.

[c] Plasma control sample.

[d] Urine control sample (10 μl).

[e] Urine control sample (50 μl).

bine (DNVB) (see below) found in urine, whereas VBL partially coeluted with this metabolite. Vindesine can also be used as the I.S., but it was not chosen because its use would increase the total analysis time.

Validation of the assay

An acceptable accuracy was achieved by using two ranges for the calculation of calibration curves. The reproducibility of the assay in plasma was tested over nine separate days, and the re-

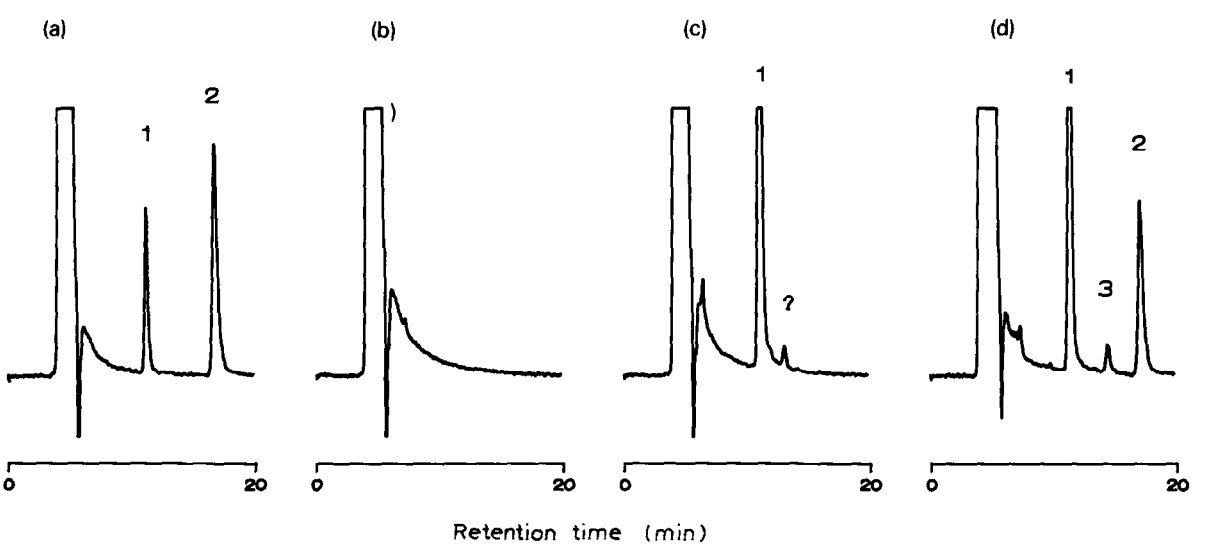

Fig. 2. Chromatograms of a plasma sample spiked with NVB (50 μg/l) (a), a patient's sample before (b) and 5 min after cessation of (c) the infusion of 30 mg/m^2 NVB and a urine sample (10 μl) collected from 0 to 24 h (d). For demonstrating the selectivity, no I.S. was added to samples b and c. Peaks: 1 = NVB; 2 = DVBL (I.S.); 3 = DNVB; ? = impurity. Detector sensitivity, 8 μA full scale.

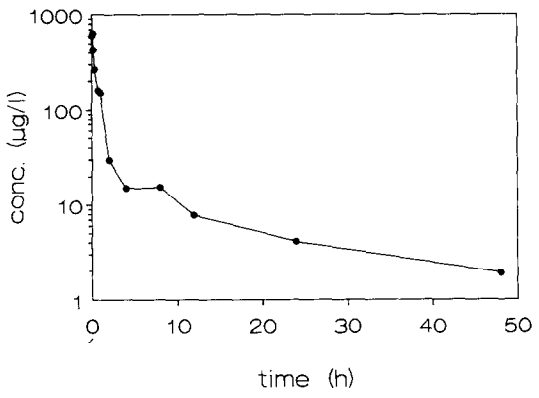

Fig. 3. Plasma concentration *versus* time curve of NVB after a 1-h intravenous infusion of 30 mg/m^2.

sults are shown in Table I. Urine samples were assayed on four days. The reproducibility for high concentrations was good, and acceptable results were obtained for concentrations near the detection limit. The minimum detectable concentration for NVB was 1.5 µg/l in plasma (500-µl sample) and 15 µg/l in urine (50-µl sample) (signal-to-noise ratio 3:1).

The applicability of this assay was demonstrated in a pharmacokinetic study in a patient suffering from a small cell carcinoma of the lung, receiving 30 mg/m^2 NVB as a 1-h intravenous infusion. Typical chromatograms are shown in Fig. 2. In plasma samples taken shortly after cessation of infusion another small peak was present next to the NVB peak. This peak was shown to represent a minor impurity of the parent drug, as it was also present in the pharmaceutical formulation. Apart from the NVB peak no other peaks were present in plasma, whereas in urine samples a secondary peak was found, which was assumed to represent DNVB. Because pure DNVB was not available, this assumption was based on its chromatographic behaviour and on its formation from NVB under conditions known to produce DVBL from VBL. Its longer retention time compared with NVB corresponds with an increased polarity of the molecule. It was the only metabolic peak detected after incubation (37°C) of NVB with FVB mice liver homogenate, and it was the major product found after overnight heating (80°C) in 0.25 *M* Na_2CO_3 buffer (pH 10.5) [15]. The plasma concentration *versus* time curve for NVB is depicted in Fig. 3.

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