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High-performance liquid chromatographic determination of vinblastine, 4-O-deacetylvinblastine and the potential metabolite 4-O-deacetylvinblastine-3-oic acid in biological fluids

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ABSTRACT

Procedures for the determination of vinblastine (VBL), 4-O-deacetylvinblastine (DVBL) and 4-O-deacetylvinblastine-3-oic acid (DVBLA) in biological samples using high-performance liquid chromatography (HPLC) combined with selective sample clean-up are presented. VBL and DVBL were determined in plasma and urine using ion-exchange normal-phase HPLC with fluorescence detection. The limit of detection was 1 μg/l for both compounds using a 500-μl sample. Successful chromatographic analyses of DVBLA were achieved by using a glass column packed with 5-μm Hypersil ODS and acetonitrile–0.05 *M* phosphate buffer (pH 2.7) (23:77, v/v). Positive identification was supported by the use of diode-array detection. The limit of detection (at 270 nm) was 10 μg/l using 1-ml samples.

INTRODUCTION

Vinblastine (VBL) (Fig. 1) and vincristine (VCR) are naturally occurring vinca alkaloids derived from the periwinkle plant *Catharantus roseus* G. Don and are active in a variety of human neoplastic disorders. It is generally believed that the interaction with microtubular protein is related to the cytotoxic action. In order to improve the therapeutic efficacy and/or to change the antitumour range of this class of com-

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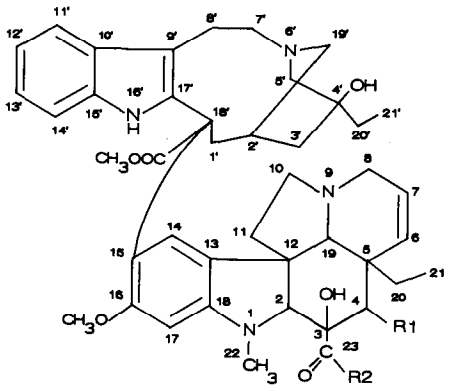

	R1	R2
VBL	$OCOCH_3$	OCH_3
DVBL	OH	OCH_3
DVBLA	OH	OH

Fig. 1. Structures of VBL, DVBL and DVBLA.

pounds, a diverse array of analogues have been developed. Vindesine (VDS) was the first of these semi-synthetic derivatives shown to be clinically useful. Other analogues are currently under clinical or preclinical investigation [1–5].

Several high-performance liquid chromatographic (HPLC) methods have been described for the determination of vinca alkaloids in plasma even at very low concentrations (1 μg/l) [6–10]. Most studies provided data on the pharmacokinetics of the parent vinca alkaloid under investigation. However, these studies were not focused on the bioanalysis of metabolic conversion products. Available information on this subject originates from older papers and is restricted to VBL, VCR and VDS only. Indications for possible metabolic conversion have been obtained with the aid of radioactively labelled drugs but without further structural elucidation [11–13]. Sethi *et al.* [14] provided data on the presence of deformylvincristine in urine from a patient who had received vincristine, and Owellen *et al.* [15] identified 4-deacetylvinblastine (DVBL) in stool and urine from patients receiving VBL.

A knowledge of the metabolic fate of vinblastine and other vinca alkaloids is essential to extend the insight into the disposition of the drug and its metabolites and clinical activity and toxicity. With vinca alkaloids, metabolites may contribute to an important extent to the antitumour activity, *e.g.*, Owellen *et al.* [15] reported DVBL to be an even more active compound than VBL in the *in vitro* test systems that they used. Apart from the 4-acetyl group of the VBL molecule, the 3- and 18′-methyl ester groups might also be susceptible to metabolic conversion to the respective carboxylic acids.

This paper is the first report on the bio-analysis of such a potential metabolic compound, *viz.*, 4-O-deacetylvinblastine-3-oic acid (DVBLA), in biological fluids.

Other theoretical metabolites (*e.g.*, vinblastine-3-oic acid and vinblastine-18-oic acid) are not yet available. Some preliminary data concerning the search for DVBL and DVBLA metabolites in human plasma and urine samples from cancer patients receiving VBL are presented.

EXPERIMENTAL

Reagents

The vinca alkaloids VBL, DVBL, DVBLA and N-(deacetyl-O-4-vinblastoyl-23)-L-ethyltryptophan (vintriptol, VtrpE) were provided by Medgenix (Brussels, Belgium) and VDS was obtained commercially. All other reagents were purchased from Merck (Darmstadt, Germany) and were of analytical-reagent grade, except for acetonitrile and chloroform, which were of HPLC-grade. Water was purified with a Mille-Q system (Millipore–Waters, Milford, MA, U.S.A.).

Instrumentation

The chromatographic analyses were performed using an HPLC system consisting of a Spectroflow SF400 pump, a Model 1000S photodiode-array detector, a Model SF980 fluorescence detector (all from Kratos, Ramsey, NJ, U.S.A.) and an MSI 660 autosampler (Kontron, Basle, Switzerland). Peak recording and integration were done on a WINNER-4 data station (Spectra-Physics, San Jose, CA, U.S.A.). Spectral data analysis was achieved on an IBM-compatible computer provided with Lab Calc software Galactic Industries, Salem, NH, U.S.A.).

Determination of VBL and DVBL

The procedures for the determination of VBL and DVBL were similar to those described previously for the investigational cytotoxic semi-synthetic derivative of VBL, N-(deacetyl-O-4-vinblastoyl-23)-L-ethyl isoleucinate (VileE) [9]. Standards solutions of VBL and DVBL were prepared by diluting stock solutions (1 mg/ml) directly with the appropriate matrix (plasma or urine). Calibration graphs were constructed in the range 1–1000 μg/l for VBL and 1–100 μg/l for DVBL.

A mixture of 500 μl of plasma or urine, 10 μl of internal standard (10 mg/l of VtrpE in acetonitrile) and 2.5 ml of 0.5 *M* phosphate buffer (pH 4.0) was extracted with 5 ml of chloroform in a glass tube. The tubes were mixed for 10 min, followed by centrifugation for 10 min at 2500 *g*. The organic layer was transferred to a clean tube and evaporated to dryness under nitrogen (37°C). The residue was dissolved in 100 μl of acetonitrile and an aliquot of 80 μl was analysed using a stainless-steel column (250 × 2 mm I.D.) packed with 5-μm Spherisorb Si. The mobile phase was acetonitrile–0.01 *M* citrate buffer (pH 3.0) (85:15, v/v) containing 0.01 *M* tetrabutylammonium bromide. The flow-rate was maintained at 0.2 ml/min. Fluorescence detection was used with excitation at 270 nm, the emission being monitored using a 320-nm long-pass filter. Ratios of the peak height of VBL or DVBL to that of the internal standard were used for quantitative calculations.

Determination of DVBLA

In glass tubes fitted with PTFE-covered screw-caps (Renes, Zeist, The Netherlands), 5 ml of 0.5 *M* phosphate buffer (pH 7.5) were added to 1000 μl of plasma. For

urine samples 1 ml of 1.0 *M* phosphate buffer (pH 9.0) was added to 1000 μl of samle. Next 10 μl of internal standard solution (20 mg/l of VDS in acetonitrile) and 5 ml of chloroform were added and the tubes were mixed for 10 min. After centrifugation for 10 min at 2500 *g*, the aqueous layer was discarded and the organic layer was transferred into a polyethylene Pony vial (Packard Instruments, Groningen, The Netherlands). A volume of 1000 μl of 0.05 *M* phosphate buffer (pH 2.5) was added and the vials were gently mixed for 5 min and subsequently centrifuged for 5 min at 2500 *g*. A volume of 700 μl of the aqueous phase was transferred into to a Model 3813 sample vial (Eppendorf, Hamburg, Germany) and lyophilized. The residue was dissolved in 100 μl of acetonitrile–water mixture (20:80).

An aliquot of 50 μl of this sample was analysed using a glass column (100 × 3 mm I.D.) packed with 5-μm Hypersil ODS preceded by a guard column (10 × 3 mm I.D.) packed with pellicular reversed-phase material (Chrompack, Middelburg, The Netherlands). The mobile phase was acetonitrile–0.05 *M* phosphate buffer (pH 2.7) (23:77, v/v). An amount of 2.5 g of tetramethylammonium chloride was added to 1 l of the mobile phase. The flow-rate was maintained at 0.4 ml/min. For quantitative purposes UV detection at 270 nm was used. Ratios of the DVBLA and VDS peak areas were used for quantitative analysis.

Patients' samples

Urine and heparinized blood samples were collected shortly before and *ca.* 1 h after administration of VBL. Plasma was obtained by centrifugation for 5 min at 2500 *g*. Plasma and urine samples were stored at −20°C until analysis within 4 weeks. Patient 1 (male, 40 years old, 70 kg) received intravenously (i.v) 6 mg/m^2 of VBL together with doxorubicin (65 mg/m^2) and bleomycin (10 mg/m^2). Patient 2 (male, 30 years old, 72 kg) also received i.v. 6 mg/m^2 of VBL together with epirubicin (70 mg/m^2), bleomycin (10 mg/m^2) and orally prednisone (40 mg/m^2).

RESULTS

Because solutions of vinca alkaloids in water are not stable, all dilution steps made during the preparation of the calibration graph should be done in plasma or urine. The detection limits of the procedure for VBL and DVBL were 1 μg/l for both compounds and a linear concentration response curve ($r > 0.99$) was obtained up to 1000 μg/l. The recoveries of extra VBL and DVBL added to patients' samples (plasma and urine) did not differ significantly from 100%. Typical within-day relative standard deviations (R.S.D.s) (calculated from the variation found between duplicates) for plasma samples were 6.7% for VBL and 7.3% for DVBL ($n = 70$), and the day-to-day R.S.D.s were 7.8% for VBL and 8.4% for DVBL ($n = 8$). For urine samples the within-day R.S.D.s were 2.5% for VBL and 3.2% for DVBL ($n = 32$) and the day-to-day R.S.D.s were 4.5% for VBL and 4.4% for DVBL ($n = 7$).

HPLC of DVBLA was performed using a Hypersil ODS column in combination with an acidic mobile phase. The peak tailing of VDS was reduced by adding tetramethylammonium chloride to the mobile phase. A linear concentration response curve for DVBLA diluted in acetonitrile–water (20:80) was obtained down to 20 μg/l, which was the minimum detectable concentration (50 μl injected, signal-to-noise ratio = 3:1). However, when DVBLA was diluted in 0.05 *M* phosphate buffer (pH 2.5) in

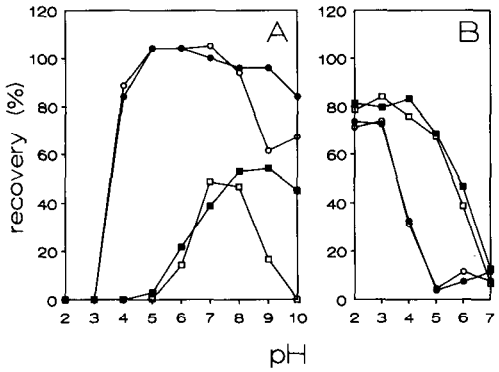

Fig. 2. (A) Recoveries of DVBLA and VDS (1 mg/l each) extracted from plasma or urine. 1000 μl of plasma diluted with 5 ml of 0.5 *M* phosphate buffer or 1000 μl of urine diluted with 1 ml of 1.0 *M* phosphate buffer were extracted with 5 ml of chloroform. The pH of the buffer ranged from 2 to 10. (B) Recoveries of the back-extraction of DVBLA and VDS. Blank plasma and urine samples diluted with phosphate buffer of pH 7.5 and 9, respectively, were extracted with chloroform. The organic phases were spiked with DVBLA and VDS and re-extracted with 0.05 *M* phosphate buffer (pH 2–7). The highest recovery in the aqueous phase was attained at low pH. ○ = VDS, plasma; ● = VDS, urine; □ = DVBLA, plasma; ■ = DVBLA, urine.

Pony vials a detection limit of 30 μg/l was achieved, with a concentration response curve that showed some upward curvature, probably caused by adsorption to the materials used.

Fig. 2 depicts extraction efficiencies of DVBLA and VDS from plasma and urine samples diluted with phosphate buffer. The extraction recoveries depended on the pH of the buffer and the optimum pH was slightly different for plasma and urine.

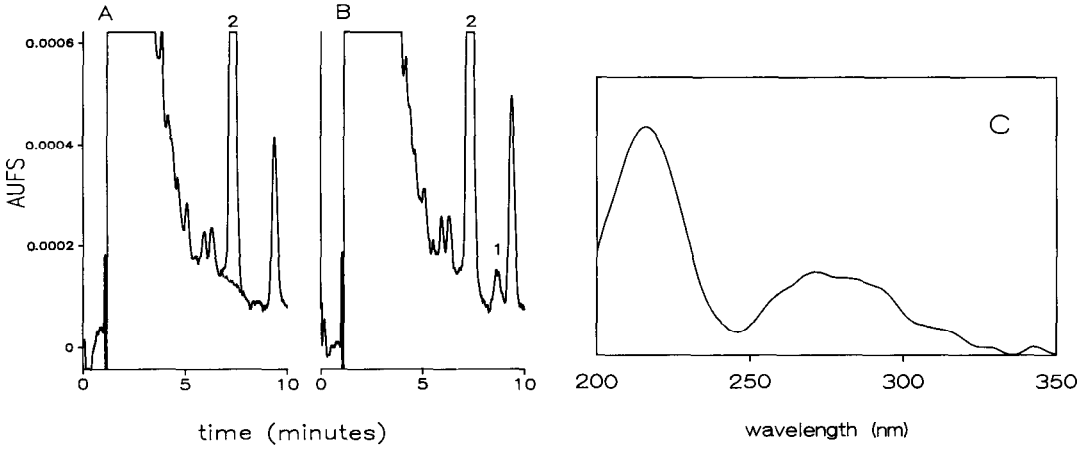

Fig. 3. HPLC of DVBLA. (A) Chromatogram of a blank urine sample [2 = VDS (internal standard; I.S.)]. The broken line represents the chromatogram without any I.S. added. (B) Chromatogram of the same specimen spiked with 20 μg/l of DVBLA. 1 = DVBLA; 2 = VDS (I.S.). (C) UV spectrum of the DVBLA peak from the urine sample spiked with 20 μg/l.

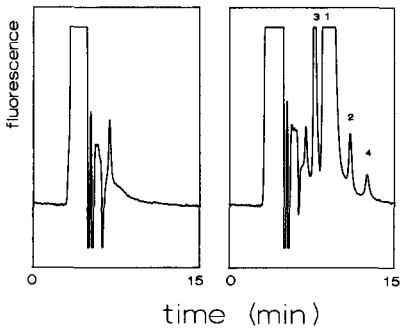

Fig. 4. HPLC of VBL and DVBL in urine. (A) Chromatogram of a urine sample from patient 2 collected shortly before administration of VBL. (B) Chromatogram of a urine.sample from patient 2 collected 1 h after administration of VBL. 1 = VBL; 2 = DVBL; 3 = VtrpE (I.S.); 4 = unknown.

The recoveries, obtained by back-extraction of the compounds from the organic phase into 0.05 *M* phosphate buffer, were optimum at acidic pH (2–3). With these procedures the minimum detectable concentrations in urine and plasma samples were 10 μg/l. The calibration graphs were not linear but showed a slight upward curvature similar to the concentration response curves of DVBLA diluted in 0.05 *M* phosphate buffer (pH 2.5). The within-run R.S.D. was 7.3% (concentration range 20–500 μg/l; $n = 32$) and the run-to-run R.S.D. for a sample spiked with 130 μg/l was 8.0% ($n = 4$).

Fig. 3 shows the chromatograms of a blank urine sample and a sample spiked with DVBLA (final concentration 20 μg/l). Even at these low DVBLA concentrations a useful UV spectrum corresponding to that of the reference compounds was obtained (Fig. 3C).

The plasma concentrations of VBL were 26 and 18 μg/l. No DVBL was detected in plasma. The urine concentrations of VBL were 6900 and 1510 μg/l and those of DVBL were 64 and 11 μg/l (patients 1 and 2, respectively). The chromatographic results for VBL and DVBL in urine samples from patient 2 are shown in Fig. 4. No DVBLA could be detected in either the plasma or urine from both patients.

DISCUSSION

In previous papers we described the determination of vinca alkaloids in plasma and showed that these compounds can be effectively extracted by the use of chloroform [8,9]. Analysis of these extracts using HPLC combined with sensitive and selective detection techniques (fluorescence or electrochemical detection) allowed detection down to 1–2 μg/l levels. After modification of the procedure described for VileE [9], the assay could also be used for the determination of VBL and DVBL. Dilution of samples with phosphate buffer of pH 4.0 permitted the simultaneous determination of VBL and DVBL. VtrpE was used as an internal standard because the extraction recovery at pH 4.0 was higher than that for VileE.

The determination of DVBLA was hampered by its unusual behaviour (extreme peak tailing and non-linearity) under most of the HPLC conditions tested. Only the combination of a Hypersil ODS or μBondapak C_{18} column and a mobile

phase buffered at pH 3.0 yielded useful chromatographic results. However, selective detection techniques could not be used, as the fluorescence and electrochemical activities of DVBLA are negligible under these conditions. UV detection at 270 nm could be used but lacked the selectivity required to analyse samples pretreated by simple one-step extraction. An extra clean-up step was therefore necessary. The pH dependence of the partition coefficient between the aqueous and organic phase allowed the back-extraction of DVBLA in a buffer solution of pH 2.5 with a high recovery. The high recovery of DVBLA in the aqueous phase might indicate that the overall polarity on this compound is determined more by the degree protonation of the nitrogen atoms in the molecule [N-1, pK_a = 3.8; N-6′ and N-9, pK_a = 6.6 (80°C)] [16] than by the dissociation state of the carboxylic acid (pK_a = 4.2). Samples thus obtained contained no interfering compounds even if detection in the low-UV range (210 nm) was employed, enabling reliable UV spectra to be acquired. For quantitative purposes UV detection at 270 nm was preferred, combining sufficient sensitivity with optimum selectivity.

With the proposed method we were able to show that DVBLA is probably not a major metabolic product from VBL, as no detectable concentrations were found in plasma or urine samples from patients receiving VBL. However, the number of patients and the sampling times used were very limited. Results concerning the possible formation of DVBLA as a metabolite of vinblastine or other experimental vinca alkaloids, currently under investigation, will be presented in future papers.

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