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Biodetermination of N-(deacetyl-O-4-vinblastoyl-23)-L-tryptophan, a metabolite of vintriptol, by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

The determination of N-(deacetyl-O-4-vinblastoyl-23)-L-tryptophan (vintriptol acid, VtrpA), a metabolite of the investigational semi-synthetic vinca alkaloid vintriptol [N-(deacetyl-O-4-vinblastoyl-23)-L-ethyltryptophan, VtrpE], in plasma and urine samples is described. Sample pretreatment included liquid–liquid extraction of the buffered (pH 5.0) biological samples with chloroform–2-propanol (95:5, v/v). The analyses were performed by ion-exchange high-performance liquid chromatography on normal-phase silica with fluorescence detection. The assay was applied to the analysis of samples from cancer patients who had been treated with VtrpE in a phase I clinical study. VtrpA was found to be a principal metabolite of VtrpE with up to 1.2% of the administered dose excreted in the urine.

INTRODUCTION

The vinca alkaloids vincristine (VCR) and vinblastine (VBL) (Fig. 1) originate from the periwinkle plant *Catharantus roseus* G. Don and are used as cytostatic drugs in the treatment of a variety of human neoplastic disorders. These drugs act as a mitotic spindle poison inducing metaphase arrest in dividing cells. Bone marrow depression and neurotoxicity are the dose-limiting

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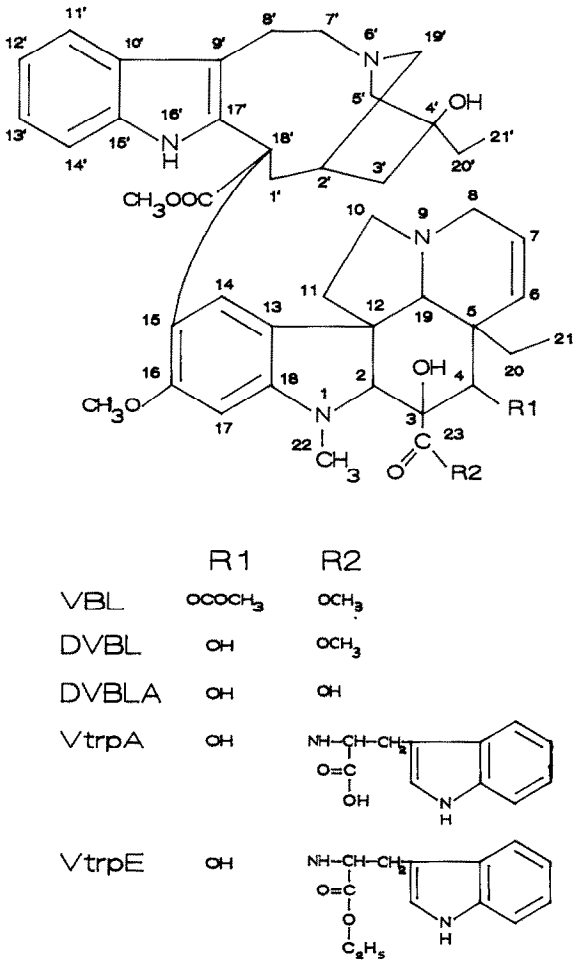

Fig. 1. Structures of vinca alkaloids. VBL = Vinblastine; DVBL = deacetylvinblastine; DVBLA = deactylvinblastine-3-oic acid; VtrpA = vintriptol acid; VtrpE = vintriptol.

factors. In the search for more effective and less toxic agents, several semi-synthetic analogues have been synthesized and tested in preclinical and/or clinical investigations [1–4]. Vindesine (VDS), which is nowadays commonly used in various single- and multi-drug regimens, was the first clinically useful semi-synthetic derivative. In a series of 21 vinblastine-23-oyl amino acid derivatives, VtrpE exhibited an increased activity, compared with VBL, against several human xenografts in mice [3]. The rationale of coupling vinca alkaloids to amino acids is that the amino acid function may facilitate the entrance of the cytotoxic compound into the tumour cells by utilizing amino acid membrane transport systems. Whether the unchanged drug is the active component or an (intracellular) metabolic conversion is required for cytotoxic action is not yet known. Potential metabolic routes for VtrpE are cleavage of the L-ethyl amino acid function, yielding 4-O-deacetylvinblastine-3-oic acid, or hydrolysis of the ethyl ester, which results in the formation of VtrpA.

VtrpE has passed phase I clinical evaluation in our hospital [4] and is now the subject of a phase II study. In an earlier paper we reported on the biodetermination and preliminary pharmacokinetics of VtrpE [5]. This method, however, was not suitable for the determination of VtrpA in biological specimens. To obtain a better understanding of the metabolic fate of VtrpE, a high-performance liquid chromatographic (HPLC) assay for VtrpA in plasma and urine was designed and used for the analysis of samples from patients treated with VtrpE. This is the first report of the determination of VtrpA in biological fluids.

EXPERIMENTAL

Reagents

VtrpE, VtrpA and VBL were obtained from Medgenix (Brussels, Belgium) and VDS from Eli Lilly (Utrecht, Netherlands). All other reagents were purchased from E. Merck (Darmstadt, Germany) and were of analytical-reagent grade, except for chloroform and acetonitrile, which were of HPLC grade. Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Instrumentation

The chromatographic analyses were performed with an HPLC system consisting of an SF400 pump, an SF980 fluorescence detector (Kratos, Ramsey, NJ, USA), a Model 360 autosampler (Kontron, Basle, Switzerland) and an AMOR electrochemical detector provided with a standard glassy carbon electrode (Spark, Emmen, Netherlands). Data analysis was done with a WINNER-4 data station (Spectra-Physics, San Jose, CA, USA).

Chromatography

Chromatographic analyses were performed by ion-exchange HPLC (HPIEC). A stainless-steel column (250 × 2 mm I.D.) was packed with 5-μm Spherisorb Si material (Phase Separations, Queensferry, UK). The mobile phase was acetonitrile–0.01 *M* sodium citrate buffer (adjusted to pH 3.0 with hydrochloric acid) (85:15, v/v) containing 0.01 *M* tetrabutylammonium bromide. The flow-rate was maintained at 0.2 ml/min. Fluorescence detection was employed with the excitation wavelength fixed at 270 nm and emission being monitored using a 320-nm high-pass filter [6].

The purity of the VtrpA peak obtained with the HPIEC method was studied by the use of a second chromatographic system based on the reversed-phase HPLC (RP-HPLC) system described in a previous paper [5]. The ratio of acetonitrile and mobile phase buffer was changed to 33:67 (v/v) to obtain an acceptable retention of VtrpA.

Sample preparation for VtrpA (method A)

For plasma, 500 μl of sample and 10 μl of a (50 mg/l) VDS solution in acetonitrile [internal standard (I.S.)] were mixed with 2.5 ml of 0.5 *M* phosphate buffer (pH 5.0), whereas for urine 500 μl of sample and 10 μl of I.S. were mixed with 0.5 ml of 1.0 *M* phosphate buffer (pH 5.0). A volume of 5 ml of chloroform–2-propanol (95:5, v/v) was added and the tubes were shaken vigorously for 10 min. After centrifugation (2500 *g*, 10 min, 4°C), the aqueous layer was discarded. The organic phase was transferred into a clean glass tube and evaporated to dryness under nitrogen (37°C). The residue was dissolved by sonication for 5 min in 100 μl of acetonitrile–water (90:10, v/v) and 50–80 μl were injected into the HPLC system.

Sample preparation for VtrpE in urine (method B)

Extraction of VtrpE from urine was identical with the procedure described for plasma [5]. A volume of 500 μl was utilized.

Calibration

Standard samples for VtrpA and VtrpE were prepared by dilution of stock solutions (1 mg/ml) in the appropriate matrix (blank plasma or urine from a healthy volunteer). The calibration samples for VtrpA in plasma ranged from 10 to 250 μg/l and for VtrpA and VtrpE in urine from 50 to 2000 μg/l.

The calibration graphs were calculated by weighted ($1/y$) least-squares linear regression analysis.

Control samples were prepared by spiking plasma or urine from different sources. Peak-height ratios between VtrpA or VtrpE and the internal standard were used for quantitative purposes.

Validation

The accuracy of the method at each standard concentration was judged from the percentage relative concentration residuals (%*RCR*), which is defined as $\%RCR = 100(RC - NC)/NC$, where *RC* and *NC* represent the interpolated and nominal concentration, respectively.

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

Stability study

The stability of VtrpE in plasma stored at −20°C was investigated by spiking blank samples with 20 or 1000 μg/l VtrpE. Aliquots were assayed together with freshly prepared specimens for a period of 20 months. The stability of VtrpE in urine (1000 μg/l) and VtrpA in plasma (250 μg/l) and urine (1000 μg/l) were evaluated for a period of 10 months.

Patients' samples

Eight patients received VtrpE as a short (2-min) intravenous infusion. Blood samples were drawn from the opposite arm before administration and 1, 5, 10, 15, 30, 45 min and 1, 2, 4, 6, 12, 18, 24 and 48 h after infusion. Plasma was sep-

arated by centrifugation and the samples were kept at −20°C until analysis (6–10 months). Urine samples were collected in 6-h portions for up to 48 h after drug administration.

RESULTS AND DISCUSSION

Chromatography

HPLC is very useful for the determination of vinca alkaloids in complex biological matrices. In order to obtain the required sensitivity and selectivity, RP-HPLC with electrochemical detection (ED) has been used [5,7,8]. However, HPIEC on unmodified silica is also very useful and provides a very selective mode of retention [6,9,10], permitting the separation of a variety of vinca derivatives with the use of only one chromatographic system (Table I). An extra advantage of HPIEC is that the large amount of organic solvent present in the mobile phase renders highly fluorescent vinca alkaloids, allowing the use of more robust fluorescence detection (FD). HPIEC was used for all quantitative analyses. Although the peak shape of vindesine was not optimum, it was suitable as an internal standard.

Sample pretreatment

In previous studies [5,6,10] it was demonstrated that many vinca alkaloids are extracted effectively form biological matrices by the use of chloroform. VtrpA (1000 μg/l) in plasma or urine diluted with phosphate buffer (1:5 or 1:1) could also be extracted with chloroform. The maximum recovery was obtained at pH 5–6, but was only about 50%. Further, the recoveries decreased at lower concentrations of VtrpA, resulting in non-linear calibration graphs and poor detection limits. Adding 5% of 2-propanol to the chloroform increased the extraction recoveries to about 80% (plasma) and 90% (urine).

TABLE I

CAPACITY FACTORS (k') OF VINCA ALKALOIDS IN THE CHROMATOGRAPHIC SYSTEM BASED ON ION-EXCHANGE NORMAL-PHASE CHROMATOGRAPHY

Alkaloid	k'
N-(Deacetyl-O-4-vinblastoyl-23)-L-ethyltryptophan (VtrpE)	1.6
N-(Deacetyl-O-4-vinblastoyl-23)-L-ethylisoleucinate (VileE)	1.6
Vinorelbine (5'-noranhydrovinblastine, navelbine) (NVB)	1.8
Vinblastine (VBL)	2.2
4-O-Deacetylnavelbine (DNVB)	2.4
4-O-Deacetylvinblastine (DVBL)	2.9
N-(Deacetyl-O-4-vinblastoyl-23)-L-tryptophan (VtrpA)	3.1
N-(Deacetyl-O-4-vinblastoyl-23)-L-isoleucinate (VileA)	4.0
Vindesine (VDS)	5.1

Validation

The limit of detection in plasma was 2 μg/l (signal-to-noise ratio 3:1). VDS was used conveniently as an internal standard, with good within-run and run-to-run reproducibility and acceptable deviations between the nominal and interpolated concentrations throughout the calibration range (Table II).

With urine samples, linear calibration graphs were obtained from 50 to 1000 μg/l with good reproducibility. Below 50 μg/l deviations from linearity were noted, but this was not a major concern, as all patients' specimens contained higher concentrations.

Stability

The stability of VtrpE and VtrpA was examined in plasma and urine stored at −20°C. Both compounds were stable over the entire test period. No conversion of VtrpE to VtrpA was observed, even after repeated freezing and thawing.

Patients' results

In a previous paper, we reported on the plasma pharmacokinetics of VtrpE [5]. Fig. 2 depicts a typical plasma concentration *vs.* time curve for VtrpA. The compound was detected in samples taken as early as 1 min after the administration of the parent drug. Its concentration rapidly declined to a minimum within 0.5 h, but then increased again, reaching maximum levels in the range 8–20 μg/l. The total urinary excretion of VtrpA was about 1% of the dose administered as VtrpE and was still present in urine samples collected after 42–48 h.

TABLE II

REPRODUCIBILITY OF THE ASSAY OF VtrpA IN PLASMA AND URINE

Matrix	Sample	Concentration (μg/l)	%*RCR*	Within-run R.S.D. (%)	Run-to-run R.S.D. (%)
Plasma[a]	Standard	10	−11.5	6.6	
		25	−4.2	2.2	
		50	−0.8	3.1	
		100	−0.1	1.5	
		250	−2.0	1.7	
	Control	21.5		4.3	7.4
Urine[b]	Standard	50	−5.8	5.1	
		100	5.0	5.1	
		200	2.4	1.4	
		500	0.1	2.2	
		2000	0.8	1.4	
	Control	255		2.5	4.9

[a] n = 8 days.
[b] n = 5 days.

The purity of VtrpA in urine samples was investigated by off-line two-dimensional chromatography. Only urine samples could be used, as they contained sufficient amounts of VtrpA for this purpose. The column effluent containing the VtrpA peak was collected, concentrated and reinjected into the chromatographic system based on RP-HPLC with electrochemical detection.

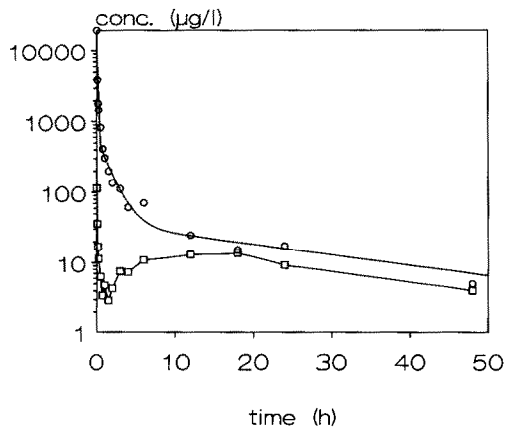

Fig. 2. Plasma concentration *vs.* time curve for (○) VtrpE and (□) VtrpA in a patient receiving 45 mg/m² vintriptol (VtrpE).

The results indicated that no interfering compounds were present under the VtrpA peak. Several batches of the parent drug were also tested for the presence of VtrpA as an impurity, and it was found that the batch, which was used for the clinical studies, contained 0.6% of VtrpA.

From these results, we conclude that the immediate appearance of VtrpA in plasma after the administration of VtrpE is caused by its presence in the parent drug formulation (0.6%). As up to 1.2% of the administered dose was recovered in urine as VtrpA and it is known that vinca alkaloids are only minimally excreted in the urine, the increasing concentration in the plasma samples up to several hours after cessation of the infusion reflects metabolic formation.

Determination of VtrpE in urine

VtrpE was extracted under the conditions described for VtrpA, but in plasma both the accuracy and the detection limits for VtrpA were negatively affected by the low recovery obtained (*ca.* 35%). In urine samples extracted at pH 3.0, the chromatograms displayed less interferences near the VtrpE peak compared with extraction at pH

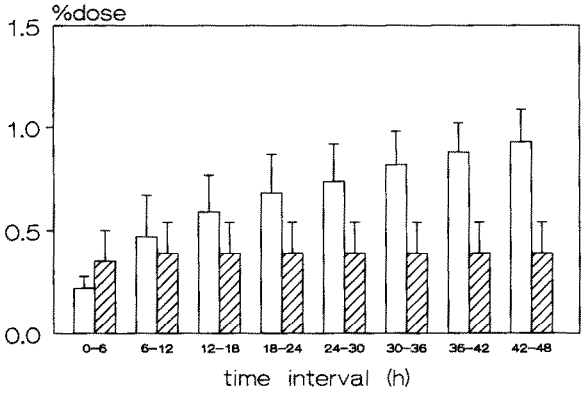

Fig. 3. Cumulative urinary excretion of VtrpA (white boxes) as percentage of the administered dose of VtrpE (hatched boxes).

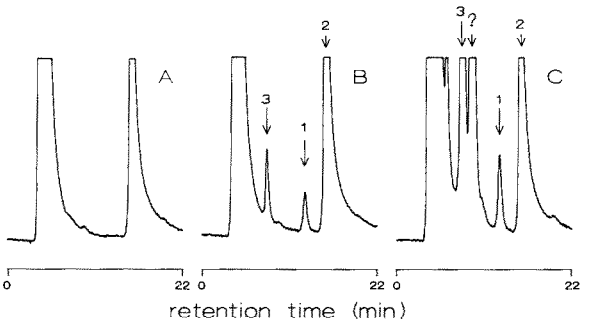

Fig. 4. Chromatograms of plasma samples. (A) Blank plasma sample; (B) plasma sample spiked with 10 μg/l VtrpA and 40 μg/l VtrpE; (C) plasma sample taken from a patient 10 min post-infusion. Next to the peaks co-eluting with (1) VtrpA, (3) VtrpE and (2) internal standard (VDS), another peak (?) representing an unknown compound was present.

5.0. Therefore, separate sample clean-up methods (A and B) were used for the determination of VtrpA and VtrpE, respectively.

Urinary excretion of VtrpE ranged from 0.2 to 0.5% of the administered dose and VtrpE was only found in the urine samples collected from 0 to 12 h after drug administration (Fig. 3).

Other metabolites

When using the combination of HPIEC and the sample pretreatment method described for VtrpA an additional plasma peak, apart from the peaks co-eluting with VtrpE and VtrpA reference compounds, was noted (Fig. 4). Quantification was not possible owing to the unknown structure and the limited amount of sample impeded further characterization. Nonetheless, based on the peak-height ratios of the metabolite to internal standard, it was concluded that the plasma concentrations and plasma clearance showed very large intra-individual variations. The compound was not detected in urine samples. Another possible metabolic product that could originate from VtrpE is 4-O-deacetylvinblastine-3-oic acid. This compound, for which we recently presented an analytical method [10], was not present in detectable concentrations (>10 μg/l) in plasma or urine samples of cancer patients treated with VtrpE.

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