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Tissue disposition, excretion and metabolism of vinblastine in mice as determined by high-performance liquid chromatography

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Abstract. We have developed and validated a selective analytical procedure, based on ion-exchange normal-phase liquid chromatography with fluorescence detection and liquid-liquid extraction, for the analysis of vinblastine (VBL) in biological matrices. The assay is suitable for the determination of the parent compound and its metabolites in plasma, tissue, faeces and urine specimens. Pharmacokinetics studies were performed in male FVB mice receiving VBL by intravenous (i. v.) bolus injection at a dose of 6 mg/kg. Plasma concentrations were monitored until 48 h after drug administration. Urine and faeces samples were collected in 24-h portions for up to 72 h and tissue samples were obtained at 4, 24, 72 and 168 h after drug administration. To facilitate a comparison between the findings we obtained by high-performance liquid chromatography (HPLC) and the results of previous studies using radiolabeled drug monitoring, some of the animals were also given radiolabeled drug. Large discrepancies were observed between the results obtained by the two methods. Excretion of the radiolabel in faeces and urine was 85% of the dose within 72 h. HPLC revealed that only 18% of the dose was excreted as unchanged drug and 19%, as measurable metabolites [O^4-deacetylvinblastine (DVBL) and two unknown compounds]. In most of the tissues taken at 4 h after drug administration, virtually all of the radioactivity represented VBL or DVBL. In all tissues taken at 72 h after drug administration, however, only very little of the radioactivity remained in the form of these compounds. Following the administration, VBL and DVBL were distributed extensively to most tissues. Many tissues appeared to possess effective means of extruding the cytotoxic drug with decreasing plasma levels. However, in some organs, including those from the genital tract and lymphatic tissues, VBL and DVBL were retained for prolonged periods. Our studies confirm previous indications that selective retention may be the basis of the activity of VBL against malignant transformations derived from these tissues.

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Introduction

The vinca alkaloids vinblastine (VBL, Fig. 1) and vincristine are naturally occurring compounds isolated from the periwinkle plant *Catharanthus roseus* G. Don. These drugs constitute an important class of anti-neoplastic agents as they are part of a number of potentially curative multi-drug regimens. Although these agents have been used for over three decades, knowledge about their pharmacokinetic behavior, tissue disposition, metabolism and excretion remains limited. Thus far, due to the absence of selective and sensitive high-performance liquid chromatography (HPLC) methodologies, all studies of tissue disposition have been executed by the administration of radiolabeled drug and subsequent quantification of the amount of radioactivity in a selected set of specimens [1–6, 8, 13]. However, the results obtained with this radiolabeled drug assay have only a limited value, as such an assay is incapable of distinguishing between the parent drug and other sources of radioactivity (e. g. metabolites or tritiated water). Combinations with thin-layer chromatography [2–4] or HPLC [1, 5, 8, 13] to determine the amount of unchanged drug and metabolites have been described. Although a thorough validation of these methods has not been obtained and their use has mainly been restricted to urine [1, 2, 4, 8] and bile samples [1–3, 8, 13], they have indicated substantial metabolism.

We have recently described a selective and sensitive HPLC method for the analysis of VBL and its metabolite DVBL in plasma [9]. We adapted our HPLC method for the quantification of VBL and its metabolites in tissue, faeces and urine samples and used it for a comprehensive pharmacokinetics study in mice. To facilitate a comparison of our findings with the results of previous studies, some animals were also given radiolabeled VBL.

Materials and methods

Drugs and chemicals. VBL and DVBL sulphate and vintriptol methane sulphonate (VtrpE) were obtained from the Medgenix Group (Fleurus,

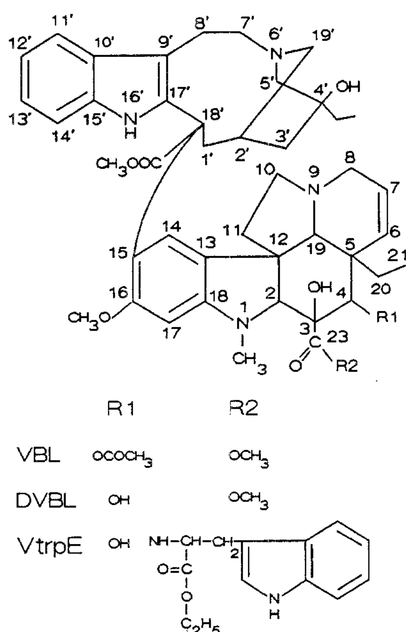

Fig. 1. Molecular structures of VBL, DVBL and VtrpE

Belgium). VBL was dissolved in 5% dextrose to obtain a final concentration of 1 mg/ml. The purity of the drug was checked by HPLC and was found to be higher than 98%. [G-^{3}H]-VBL sulphate (specific activity, 338 MBq/mg) dissolved in ethanol was obtained from Amersham International (Buckinghamshire, UK). Unlabeled VBL was dissolved in 1 ml ethanol and radiolabeled VBL was added. The combined mixture was evaporated under nitrogen (37° C) and the residue was dissolved in 5% dextrose to obtain a final concentration of 1 mg/ml. As verified by HPLC, about 97% of the radiolabel co-eluted with VBL. All other reagents were purchased from E. Merck (Darmstadt, Germany) and were of analytical quality except for acetonitrile, which was of HPLC grade. Blank human plasma was obtained from healthy donors. Water purified by the Millipore-Q system (Waters, Milford, Mass., USA) was used throughout the experiments.

Animal study. All experiments were performed in male FVB mice aged 6–10 weeks, which were given food and water ad libitum. The drugs were given as an i. v. bolus injection (6 mg/kg) in the tail vein (average injection time, 5 s).

Tissue disposition. Tissues from four to six animals per time point were removed at 4, 24, 72 and 168 h after drug administration. These included brains, muscle (from the back), abdominal fat, stomach, small and large intestine, appendix, liver, gall-bladder, kidneys, lungs, spleen, heart, testis plus epididymis, thymus, peripheral and mesenteral lymph nodes and plasma. Three mice received radiolabeled drug. Their organs were resected at 4, 24 and 72 h, respectively. Tissues were homogenized by means of a tissue homogenizer (Biospec Products, Bartlesville, Okla., USA) at 4° C in 1–7 ml blank human plasma (approximately 0.1–0.2 g tissue/ml) and stored at –20° C until analysis.

Excretion studies. Seven animals were placed in metabolic cages. Three of them were given radiolabeled VBL, and the other four received only the unlabeled drug. Their urine and faeces were collected in 24-h portions for a total period of 72 h. The faeces was homogenized, using the tissue homogenizer, in 10 ml 4% (w/w) bovine serum albumin (BSA; Organon Technika, Boxtel, The Netherlands) in water. Faeces and urine samples were stored at –20° C.

Drug analyses. The analytical procedures used for the quantification of the drug levels in plasma have been described in detail elsewhere [9]. Slightly modified procedures were used for the quantification of drug in the other biological matrices. Thawing of the samples always took place at 15°–20° C in a water bath. A volume of 200 or 500 µl homogenized tissue sample was pipetted into glass tubes equipped with a Teflon-covered screw cap and completed to 500 µl with blank human plasma. For quantification in urine, a volume of 10 or 50 µl urine sample and 500 µl blank human plasma was used, whereas for faeces specimens, 100 µl faeces homogenate and 500 µl blank plasma was used. VtrpE (50 µl; 2 mg/l in acetonitrile) was used as the internal standard solution (IS), and extraction was carried out with 4 ml diethyl ether. The tubes were shaken for 10 min and then centrifuged for 5 min at 1,500 *g* (4° C). The aqueous layer was frozen in ethanol/solid carbon dioxide and the organic phase was decanted into a clean tube and evaporated to dryness under nitrogen (37° C). The residue was dissolved in 100 µl acetonitrile by sonication for 5 min and an aliquot of 80 µl was subjected to chromatography.

The HPLC system consisted of a Spectroflow SF400 pump, a Spectroflow 980 fluorimetric detector, a 1000S UV-Diode Array Detector (Kratos, Ramsey, N.J., USA) and a Model 360 autosampler (Kontron, Basel, Switzerland). Chromatographic analyses were performed on a stainless steel column (250 × 2 mm) packed with 5 µm Spherisorb Si material (Phase Separations, Queensferry, UK). The mobile phase comprised a mixture of acetonitrile and 10 m*M* citrate buffer (pH 3.0; 85/15, v/v) and contained 10 m*M* tetrabutylammonium bromide. Elution was carried out at ambient temperature with a flow rate of 0.2 ml/min. The excitation monochromator was set at 270 nm, whereas emission was monitored using a 320-nm long-pass filter. Integration was carried out using a WINner-4 data station (Spectra Physics, San Jose, Calif., USA).

Calibration. Calibration curves constructed from spiked human plasma in the range of 5–500 ng/ml were used for the quantification of drug in the plasma, urine and tissue samples. The concentration in faeces specimens was calculated from a separate calibration curve, also constructed in plasma, but with the addition of 100 µl blank faeces homogenate to each calibration sample. Ratios of peak areas of analytes and internal standard were used for quantitative purposes.

Validation. The accuracy of the method in the various biological materials was determined in blank samples spiked with VBL and DVBL. The specimens were analysed over 3 separate days, with repeated freezing and thawing of the samples being carried out. The precision was determined from samples derived from animals receiving VBL. Tissue specimens were obtained from animals at 4 h after the administration of 1 or 6 mg/kg VBL, and homogenates containing approximately 50 ng/ml were selected. For urine and faeces, specimens obtained between 0 and 24 h were used. All samples were assayed during several analytical runs to serve as control samples.

Radiolabeled drug assay. Volumes of 200 µl injection fluid (diluted 1 : 1,000 with acetonitrile), 200 µl tissue homogenate, 100 µl faeces homogenate or 50 µl urine specimen were mixed with 10 ml Ultima Gold scintillation fluid (Packard Instrument Company, Meriden, Conn., USA). Radioactivity was determined in a Tri-Carb Series 4000 Minaxi model B4430 liquid scintillation counter (Packard Instrument Company, Meriden, Conn., USA) and values were corrected for quenching by means of external standardization.

Results and discussion

Analytical procedure

The HPLC assay used in this study was initially developed for the quantification of VBL and DVBL in plasma samples [9]. To enable drug quantification in other biological matrices, some adaptations were made. Sample handling procedures are an essential part of an analytical method for the determination of vinca alkaloids in biological

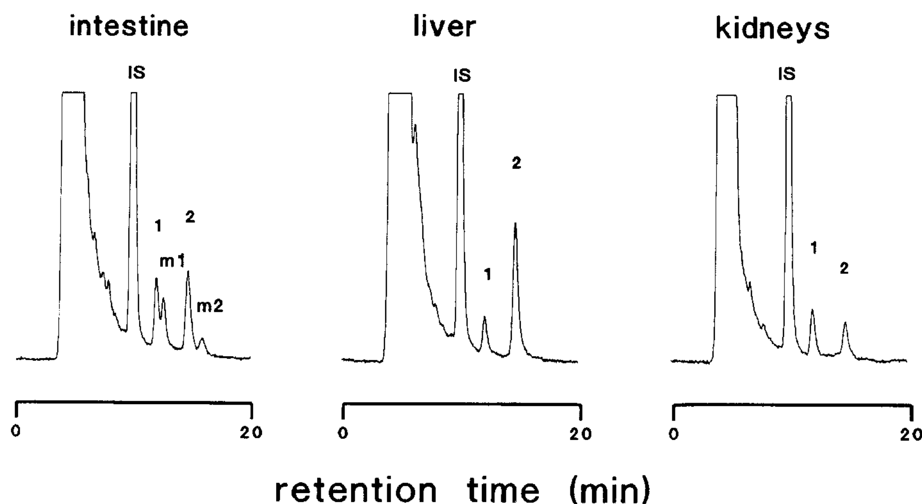

Fig. 2. Chromatograms of tissue samples taken at 24 h after drug administration. *1*, VBL, *2*, DVBL, *IS*, internal standard (VtrpE); *m1*, *m2*, unknown metabolites

Table 1. Accuracy of the assay in spiked tissue samples

Sample	Concentration spiked (ng/ml)	VBL (mean ± SD)	DVBL (mean ± SD)
Brains	50	120.3% ± 10.5%	124.5% ± 6.1%
Muscle	50	97.4% ± 3.0%	102.5% ± 5.2%
Abdominal fat	50	67.4% ± 10.9%	87.2% ± 4.4%
Colon	50	107.7% ± 6.6%	104.3% ± 7.2%
Appendix	50	106.8% ± 7.2%	104.1% ± 5.6%
Large intestine	50	97.9% ± 0.2%	104.9% ± 4.4%
Stomach	50	101.5% ± 11.5%	103.9% ± 7.1%
Liver	50	90.9% ± 10.0%	125.5% ± 7.9%
Kidneys	50	106.6% ± 8.0%	108.9% ± 8.3%
Lungs	50	93.7% ± 6.2%	100.8% ± 5.1%
Spleen	50	99.9% ± 5.8%	106.1% ± 4.8%
Heart	50	97.7% ± 6.4%	102.2% ± 8.0%
Testis	50	98.5% ± 6.2%	103.3% ± 6.0%
Epididymis	50	84.0% ± 3.1%	94.3% ± 6.6%
Thymus	50	89.8% ± 5.5%	92.7% ± 7.1%
Lymph nodes[a]	50	93.2%	102.6%
Plasma	50	96.4% ± 4.2%	101.5% ± 3.4%
Urine	500	84.7% ± 5.1%	90.3% ± 6.7%
	10,000	89.2% ± 6.5%	98.2% ± 7.3%
Faeces	250	88.9% ± 12.8%	91.7% ± 8.9%
	5,000	89.9% ± 10.8%	89.3% ± 7.8%

The accuracy of the method was determined in blank samples spiked with VBL and DVBL and assayed over three separate analytical runs

[a] Analysed on 1 day only

Table 2. Precision of the assay as calculated from samples derived from animals receiving VBL, which were used as control samples

Sample	VBL (mean ± SD)[a]	RSD	*n*
Brains	6.6 ± 1.4	21.6%	5
Muscle	40.7 ± 1.1	2.6%	5
Abdominal fat	6.0 ± 1.0	16.0%	4
Colon	16.9 ± 2.0	12.0%	4
Appendix	17.1 ± 1.5	9.1%	4
Large intestine	34.0 ± 5.1	14.9%	5
Stomach	36.9 ± 3.4	9.3%	4
Liver	37.3 ± 4.2	11.4%	9
Gall-bladder	23.7 ± 2.1	9.0%	4
Kidneys	49.5 ± 3.8	7.6%	9
Lungs	84.3 ± 6.8	8.1%	4
Spleen	49.5 ± 3.1	6.2%	4
Heart	34.0 ± 4.0	11.7%	4
Epididymis	46.8 ± 3.6	7.6%	6
Urine	7,578 ± 427	5.6%	3
Faeces	2,408 ± 392	16.3%	6

[a] Mean concentration (ng/ml) in the tissue homogenate

n, Number of analytical runs; RSD, Relative Standard Deviation

specimens and have previously been discussed extensively [10]. Modification of our 'plasma assay' for urine and tissue samples was easily achieved by adding a limited portion of the unknown specimen (≤ 50 µl urine or ≤ 500 µl tissue homogenate) to a tube containing 500 µl blank human plasma and processing this sample further as if it were a plasma sample. This procedure allowed calibration curves to be constructed in plasma and to be used for reading the concentration of these samples with acceptable analytical accuracy (Table 1). Some accuracy problems were encountered with the analysis of faeces, because the addition of 100 µl faeces suspension decreased the absolute recovery of VBL, DVBL and internal standard in a non-proportional manner. An acceptable accuracy for faeces specimens was obtained when separate calibration curves (also constructed in plasma) were used with each standard sample containing a volume of 100 µl blank faeces homogenate. The day-to-day reproducibility in faeces specimens was also somewhat higher, which was probably related to the difficulty of obtaining a good homogeneous faeces sample (Table 2). For minimization of the erroneous results caused by non-representative sampling, the average result obtained in every faeces sample analysed during three separate analytical runs was used.

Water has been recognized as an unfavorable matrix for vinca alkaloids because of the loss of analyte due to its adsorption to the wall of the test tube [10]. This phenomenon does not occur in the presence of sufficient (40 g/l) albumin. Therefore, BSA was used as a solution for the

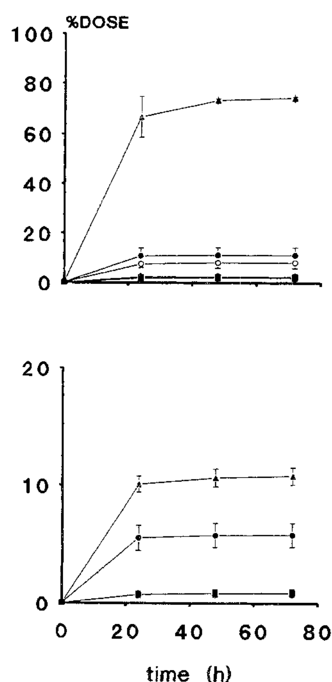

Fig. 3. Excretion profiles of total radioactivity (▲), VBL (●), DVBL (■), M1 (○) and M2 (□) in faeces (*upper plot*) and urine (*lower plot*). *Error bars* indicate the SD

homogenization of faeces. Initially, tissues were also homogenized in BSA. However, it appeared that in most tissues the concentrations found in spiked control samples declined upon freezing and thawing. These problems did not occur when the tissues were homogenized in human plasma; the specimens remained stable even after repeated freezing and thawing.

Pharmacokinetics studies

Representative chromatograms for the analysis in liver, kidneys and large intestines are depicted in Fig. 2. The chromatograms obtained using these procedures were free of interfering peaks, allowing accurate and sensitive quantification of VBL and its metabolites. Apart from VBL and DVBL, the chromatographic results revealed the presence of two other compounds (M1 and M2). Both of these compounds were found in tissues of the gastro-intestinal tract (predominantly in the appendix) and in faeces but not in organs of the central compartment (liver, kidneys) or in any other tissues. These compounds were also not found in urine. Due to the relatively small quantities excreted, isolation for structure elucidation was not considered. On the basis of their retention times, the two unknown compounds M1 and M2 appear to be closely related to VBL and DVBL, respectively. By the use of on-line UV-diode array detection, their spectra were superimposable with those of VBL and DVBL. From these observations we assume that these compounds are formed intra-intestinally and that identical structural alterations are introduced in both VBL and DVBL. Due to their absence in plasma and tissues of the central compartment, they are probably not relevant for

Table 3. Recovery of radioactivity in the aqueous phase after liquid-liquid extraction

	Recovered radioactivity	
	$t = 0$	$t = 24$ h
Spiked specimens:		
Plasma	12.2%	13.2%
Urine	14.0%	14.3%
Faeces	20.4%	28.3%
Murine specimens:		
Urine	20.4%	
Faeces	56.2%	

Biological samples were extracted with diethyl ether and the amount of radioactivity recovered in the aqueous layer was determined as a percentage of the total radioactivity present. Both blank samples spiked with [^{3}H]-VBL and samples collected from mice at 0–24 h after drug administration were used. To test the stability of the tritium label at in vivo temperatures, spiked samples were also incubated in a water bath for 24 h at 37° C

the anti-proliferative effects. Their occurrence in humans needs to be evaluated, as they may play a role in the gastro-intestinal toxicity of VBL. The concentrations of M1 and M2 were calculated from the standard curves of VBL and DVBL, respectively.

The total amount of VBL, DVBL, M1 and M2 excreted in faeces and urine over a 72-h period accounted for 37% of the delivered dose (Fig. 3). During this period the total amount of radioactivity recovered was 85%, which corresponds to the results reported for previous studies using radiolabeled drugs [2, 3, 8]. After liquid-liquid extraction with diethyl ether of the faeces specimens (0–24 h) obtained from animals receiving radiolabeled VBL, the radioactivity remaining in the aqueous fraction was 52.6%, whereas the corresponding value for blank faeces samples spiked with radiolabeled drug was 20.4% (Table 3). These results indicate the occurrence of a considerable amount of very polar and, therefore, presumably inactive conversion products. The recovery of radioactivity in the effluent after HPLC analysis of the ethereal fraction with subsequent fractionated collection and off-line scintillation counting was quantitative after 20 min, indicating the absence of any compound remaining on the column. Counting of the serial fractions of the column effluent showed that a substantial part (approximately 30%) of the radioactivity eluted as a broad band in the early region (retention time, ≤ 8 min) of the chromatogram (Fig. 4). This chromatographic behaviour indicates that this band represents several compounds, all of which possess a decreased capability to interact with the stationary phase. Together with the observed loss of fluorescence properties, these results provide strong evidence of structural alterations in the basic catharantine-vindoline backbone of the molecule. Because such alterations are nearly always associated with a loss of cytotoxic activity, these compounds are presumably also inactive agents [11].

In tissue specimens it appeared that at 4 h after drug administration, most of the radioactivity persisted in the form of VBL and DVBL (Fig. 5). However, in tissues taken at 24 and 72 h after drug administration, a substantial

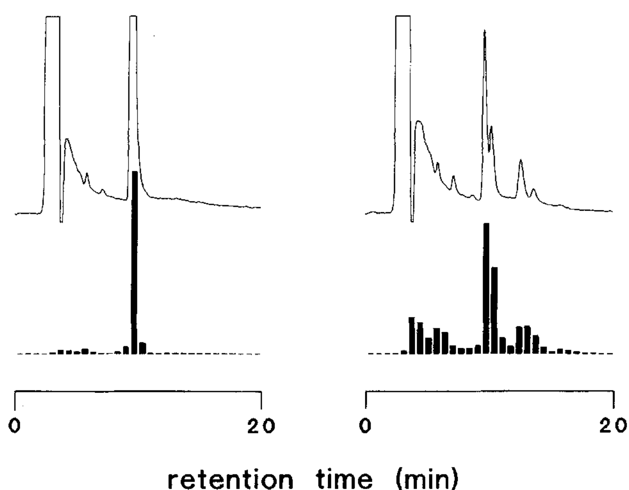

Fig. 4. Chromatograms of blank faeces specimens spiked with [^{3}H]-VBL (*left chart*) and faeces specimens collected between 0 and 24 h after the administration of radiolabeled drug to mice (*right chart*). The *upper traces* were obtained by direct fluorescence detection. The *lower results* were obtained from fractionated collection and subsequent off-line scintillation counting of the column effluent. No internal standard was added. The early peaks in the upper chromatograms are due to the presence of endogenous compounds

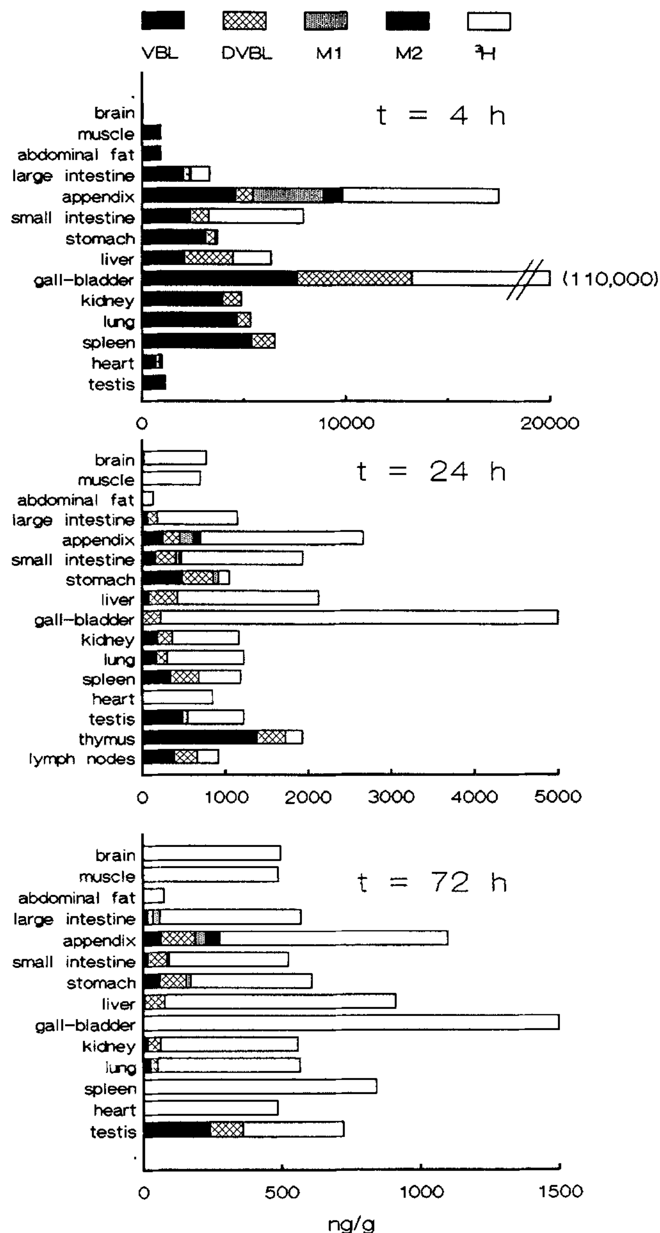

Fig. 5. Distribution of VBL and its metabolites in tissue obtained at serial time points (one animal per time point). The full length of the bars depicts the total amount of radioactivity recorded; within this bar the respective fractions of VBL, DVBL, M1 and M2 are given

and increasing fraction of the radioactivity was no longer associated with VBL or DVBL. After 72 h, the distribution of radioactivity was fairly homogeneous through all of the tissues at a concentration equivalent to approximately 600 ng VBL/g tissue, corresponding to 10% of the delivered dose (6 mg/kg). Together with 85% of the radiolabel being excreted within 72 h, this brings the overall recovery of radioactivity to about 95% of the delivered dose.

These findings in faeces, urine and tissue samples clearly demonstrate the importance of using selective procedures for studying the pharmacokinetics of this type of compound. The extensive scale on which VBL is converted into metabolites, which represent entities that exhibit very different physico-chemical features (e. g. polarity) and, therefore, probably also decreased or lost cytotoxic properties, reduces the value of the results obtained in previous studies using non-selective radiolabeled drug assays.

In many organs, the tissue concentration of VBL was accurately reflected by the plasma concentration as indicated by the fraction of drug remaining in the tissue at 24 h after drug administration relative to that in the 4-h samples, which was less than 10% for most tissues and 5.3% for plasma (Table 4). As has been discussed in a recent review [11], a number of previous studies have provided evidence that the activity of vinca alkaloids is based on their prolonged retention within the target cell. Our pharmacokinetics study using a selective HPLC methodology demonstrates that selective retention takes place in normal tissues of testicular and lymphatic origin, whereas rapid drug extrusion occurs from most other tissues. This selective retention may explain the activity of VBL against lymphomas and testicular carcinoma in the clinical setting. Retention was also obvious in the stomach and the brains. A minor penetration of VBL through the blood-brain barrier is probably driven by the very high initial plasma levels present after i. v. bolus injection, whereas the blood-brain barrier may later serve as an obstruction to its rapid elimination from the brain.

A remarkable observation was the profound distribution and retention of DVBL (Table 5). In cell cultures this compound has been shown to possess cytotoxic activity similar to that of VBL [7]. Although DVBL is also a metabolite of VBL in humans, it is found in only minimal quantities in the urine, whereas plasma levels remain undetectable [12]. Therefore, this metabolite cannot play a role in the antitumor effect of VBL in humans.

Table 4. Tissue concentrations of VBL

Sample	$t = 4$ h	$t = 24$ h	$t = 72$ h	$t = 168$ h
Brains	78 ± 25	36 ± 6 (46%)	ND	–
Muscle	664 ± 130	ND	ND	–
Abdominal fat	564 ± 120	20 ± 15 (4%)	ND	–
Colon	3,343 ± 880	98 ± 34 (3%)	16 ± 23 (0.5%)	–
Appendix	4,430 ± 580	257 ± 157 (6%)	22 ± 31 (0.5%)	–
Large intestine	2,266 ± 400	173 ± 27 (8%)	23 ± 12 (1%)	–
Stomach	3,440 ± 520	470 ± 115 (14%)	51 ± 13 (1%)	–
Liver	2,320 ± 390	63 ± 22 (3%)	ND	–
Gall-bladder	7,270 ± 2,140	ND	ND	–
Kidneys	4,450 ± 830	141 ± 42 (3%)	ND	–
Lungs	4,860 ± 460	166 ± 30 (3%)	ND	–
Spleen	6,660 ± 1,440	679 ± 310 (10%)	21 ± 31 (0.3%)	–
Heart	680 ± 150	ND	ND	–
Testis	358 ± 39	235 ± 25 (66%)	198 ± 20 (55%)	194 ± 16 (54%)
Epididymis	1,519 ± 360	540 ± 53 (36%)	197 ± 62 (13%)	–
Thymus	2,200 ± 50	1,142 ± 175 (52%)	183 ± 19 (8%)	ND
Lymph nodes	2,435 ± 280	223 ± 97 (9%)	ND	–
Plasma	94 ± 27	5.0 ± 1.6 (5%)	ND	–

Data represent mean values ± SD expressed in ng VBL/g tissue. The fraction of drug remaining relative to that in the 4-h specimen is given in parentheses.

ND, Not detectable; –, not determined

Table 5. Tissue concentrations of DVBL

Sample	$t = 4$ h	$t = 24$ h	$t = 72$ h	$t = 168$ h
Brains	ND	ND	ND	–
Muscle	130 ± 36	ND	ND	–
Abdominal fat	43 ± 8	ND	ND	–
Colon	640 ± 200	106 ± 36 (17%)	18 ± 16 (3%)	–
Appendix	960 ± 190	226 ± 137 (24%)	40 ± 31 (4%)	–
Large intestine	900 ± 210	266 ± 67 (30%)	54 ± 12 (6%)	–
Stomach	600 ± 90	329 ± 110 (55%)	79 ± 13 (13%)	–
Liver	2,880 ± 720	273 ± 92 (10%)	45 ± 43 (2%)	–
Gall-bladder	1,804 ± 270	ND	ND	–
Kidneys	1,340 ± 360	147 ± 55 (12%)	30 ± 26 (2%)	–
Lungs	770 ± 130	101 ± 23 (13%)	ND	–
Spleen	1,490 ± 420	800 ± 224 (54%)	89 ± 73 (6%)	–
Heart	270 ± 90	ND	ND –	
Testis	30 ± 5	45 ± 6 (150%)	47 ± 6 (157%)	58 ± 5 (193%)
Epididymis	134 ± 26	137 ± 10 (102%)	94 ± 26 (70%)	–
Thymus	90 ± 5	214 ± 52 (238%)	177 ± 39 (197%)	50 ± 24 (56%)
Lymph nodes	257 ± 47	177 ± 54 (69%)	ND	–
Plasma	69 ± 12	8.3 ± 4.5 (12%)	ND	–

Data represent mean values ± SD expressed in ng DVBL/g tissue. The fraction of drug remaining relative to that in the 4-h specimen is given in parentheses.

ND, Not detectable; –, not determined

Conclusions

We studied the murine pharmacokinetics of VBL and its metabolites by using a validated, selective and sensitive HPLC procedure. The results clearly show the need for such methodologies, as vinca alkaloids are metabolized extensively, and HPLC assays should therefore be part of every future pharmacokinetic investigation of vinca alkaloids and/or semi-synthetic derivatives. Furthermore, our results strongly support the hypothesis that the prolonged retention of VBL is the basis of its antitumor efficacy.

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