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Determination of vinca alkaloids in mouse tissues by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method is described for the determination of vinblastine in various normal mouse tissues, such as lung, heart, liver, kidney and muscles, and in implanted MO_4 tumours. Vincristine was used as the internal standard. Freshly obtained mouse tissue or tumour tissue was frozen at −20°C and then lyophilized. After lyophilisation, the dry tissues were pulverized and homogeneously mixed, and an aliquot was suspended in 0.1 *M* hydrochloric acid. The drugs of interest were then isolated from this suspension using ion-pair extraction at pH 3 with octylsulphate as counter-ion. The obtained extracts were analysed on a reversed-phase system with a cyanopropyl stationary phase. The detection limit was 1 ng/l in plasma and 10 ng/g in tissue. The extraction recoveries of vincristine and vinblastine were between 45 and 67%, and there were no interferences from blank components. Preliminary pharmacokinetic data for different mouse tissues and tumour implanted in muscle tissue are presented.

INTRODUCTION

Vinblastine sulphate (VLB), vincristine sulphate (VCR) and vindesine are vinca alkaloids used in cancer chemotherapy. VCR and VLB are naturally occurring, and are obtained from the *Vinca rosea* plant. Vindesine (4-desacetylvinblastine amide) is a semisynthetic derivative of VLB. Although the molecular structures of these alkaloids are very similar, their clinical toxicity, activity and pharmacokinetic behaviour can vary widely [1].

Until 1983, most methods for monitoring vinca alkaloids were based on radioimmunoassay techniques [2], flow cytometry and enzyme-linked immunosorbent assay (ELISA) techniques [3,4].

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In the second half of the 1980s, high-performance liquid chromatographic (HPLC) techniques became available. We have previously described such methods for the determination of vinca alkaloids in plasma and urine, and in tumour cells and culture medium [5–7]. Other very important work was done by Vendrig and co-workers [8–10], who lowered the detection limit by introducing electrochemical [9] and fluorescence detection [10], making it possible to determine levels of vinblastine or vindesine below 1 ng/ml. The chromatographic analysis of vinca alkaloids and other anticancer drugs is further discussed in a review [2].

Only one method to assay vinca alkaloids in tissues has been reported [11]. It uses a combination of HPLC and radiolabeling. Radiolabeled drugs are sometimes difficult to handle, but they offer the advantage of the determination of the intrinsic recovery of the compounds. This advantage can be nullified when the HPLC technique offers a high feasibility and accuracy, as was demonstrated by Vendrig and co-workers [8–10] or De Smet and co-workers [5,6].

This paper describes a method to determine VLB in a quantitative way, based on an easy lyophilization, combined with ion-pair extraction and reversed-phase HPLC. This method allows the assay of VLB and, if necessary, other related vinca alkaloids in lung, heart, liver, kidney and muscles of CDF_1 mice and in implanted MO_4 mouse fibrosarcoma tumors.

EXPERIMENTAL

Apparatus

A Varian 2010 pump as solvent-delivery system and a Varian 2050 variable-wavelength UV detector (Varian, Palo Alto, CA, USA) operated at 220 nm were used. The attenuation of the detector was set at 0.01 a.u.f.s. The samples were injected with a Gilson 231 autosampler (Gilson, Ghent, Belgium), equipped with a 100-μl sample loop. The chromatograms were recorded and integrated with an Hitachi D-2000 integrator (Merck, Darmstadt, Germany). The column was stainless steel (250 mm × 4 mm I.D.), packed with LiChrosorb CN, particle size 5 μm (Merck). A guard column (30 mm × 4 mm I.D.) packed with the same material was used. The mobile phase was acetonitrile–phosphate buffer (pH 3, 0.04 *M*) (60:40, v/v). All analyses were performed at ambient temperature. All laboratory glassware was silanized using Surfasil (Pierce, Rockford, IL, USA), which was diluted with acetone.

Standards and reagents

VLB (Velbe) and VCR (Oncovin) were obtained from Eli Lilly (Brussels, Belgium) and were of pharmaceutical purity. Acetonitrile and dichloromethane were of liquid chromatographic grade and chloroform was pro analysis quality. They were all obtained from Merck. For the preparation of the phosphate buffer solutions, doubly distilled water, which was further purified with a Water-I system (Gelman Sciences, Ann Arbor, MI, USA), was used. Hydrochloric acid, sodium octylsulphate, phosphoric acid and sodium dihydrogenphosphate monohydrate were all pro analysis quality and obtained from Merck.

For the mobile phase, a 0.04 *M* phosphate buffer (pH 3) was used. For the ion-pair extraction, a 0.4 *M* phosphate buffer (pH 3), containing 0.05 *M* octylsulphate, was used.

Extraction procedure

The freshly obtained tissues were accurately weighed and frozen at −20°C. The tissues were lyophilized for 24 h. Each dried tissue was pulverized in a mortar and homogeneously mixed. An aliquot of this powder was taken and the extraction procedure was carried out. The following amounts of the dried tissue powder were taken: 25 mg of heart, 25 mg of kidney, 30 mg of lung, 50 mg of liver, 40 mg of muscle and 50 mg of tumour.

In order to determine the extraction recovery, 100 μl of VLB solution (5, 50 or 500 μg/ml) and 100 μl of VCR solution (5, 50 or 500 μg/ml) were added to the aliquot of dried tissue. Then 5 ml of 0.1 *M* hydrochloric acid were added to the centrifuge tube, and the sample was ultrasonicated for 30 min. To this suspension, 5 ml of acetonitrile were added dropwise by continual vortex-

mixing. After centrifugation for 30 min, the supernatant was decanted into another centrifuge tube, and the acetonitrile fraction was evaporated in a water-bath at 60°C under a gentle stream of nitrogen. Next, 10 ml of 0.4 *M* phosphate buffer (pH 3) containing 0.05 *M* sodium octylsulphate and 5 ml of chloroform were added. The samples were shaken for 30 min in a shaking bath. After centrifugation, 4 ml of the chloroform phase were pipetted into a reacti-vial and evaporated to dryness at 30°C under a gentle stream of nitrogen. The residue was dissolved in 500 μl of dichloromethane, and 100 μl were injected into the HPLC system.

For the determination of the extraction recoveries of VLB and VCR from the tissues, quantitation was performed by comparison with an unextracted standard curve in water. At least six samples of tissue were used to calculate the extraction recovery at each concentration.

Preparation of standard curves in water and tissues

Stock solutions were prepared in doubly distilled water in the following concentrations: VLB, 200 μg/ml; VCR 20 μg/ml.

A standard curve for the determination of VLB in mouse plasma was made with spiked plasma at the following concentrations: 0, 50, 100, 150, 200 and 500 ng/ml. For the determination of the extraction recoveries of VLB and VCR from different tissues, standard curves in water were prepared in the following concentrations: 0, 200, 400, 600, 800 and 1000 ng/ml. Calibration curves for vinblastine in the different tissues were obtained at the concentration 0, 2, 4, 8 and 16 μg/g tissue.

For the pharmacokinetic studies in mice, extracted curves in each tissue were made at the concentrations 0, 2, 4, 8 and 16 μg/g of tissue. VCR was used as internal standard at a concentration of 4 μg/g of tissue. Plasma standard curves were prepared in the range 5–500 ng/ml.

Cell culture and tumour implantation in CDF_1 mice

The malignant MO_4 fibrosarcoma cell line was obtained from a C_3H mouse embryo cell line by transformation with a Kirsten murine sarcoma virus [12]. Cells were maintained *in vitro* and prepared as previously described [6] and 10^6 cells were injected subcutaneously into the right inguinal region of CDF_1 mice.

Tumours were measured by callipers and their volumes were calculated according to Corbett *et al.* [13]. When the tumour volume reached 250 mm^3 (± 10%), mice were ready to be injected with VLB.

Pharmacokinetic data of non-tumour-bearing mice and of MO_4 tumour-bearing mice

Non-tumour-bearing male CDF_1 mice were selected at the age of 14 weeks (20–24 g). VLB sulphate (2 mg/kg body weight, suspended in 0.5 ml of solution) was injected as an intravenous bolus injection via the tail vein.

Four to seven mice were taken for each point of the pharmacokinetic curve. Tissue samples (lung, heart, kidney, liver and muscle) were taken 3, 5, 10, 15, 30, 60 and 90 min, and 6, 12 or 24 h after VLB injection. The sampled tissues were treated as described under *Extraction procedure*, except that only VCR as internal standard was added (100 μl of a 5 μg/ml stock solution).

A blood sample was taken for the determination of VLB in plasma. The technique for determination of VLB in plasma has been described previously [4].

Quantitation was carried out by interpolation on an extracted standard curve in the tissues of interest (peak area VLB/internal standard *versus* concentration of VLB).

The same procedure was carried out for tissues from tumour-bearing mice after the implanted tumour had reached a volume of 250 mm^3 (± 10%), except that at that moment tumour tissue was also sampled. Tumours were implanted in the right thigh muscle by insertion of the needle in the right inguinal region, at the age of 10 weeks. The muscle of the left thigh was used for sampling muscle tissue in these tumour-bearing mice.

Results were expressed in μg of VLB per g of tissue, or in μg of VLB per ml of plasma.

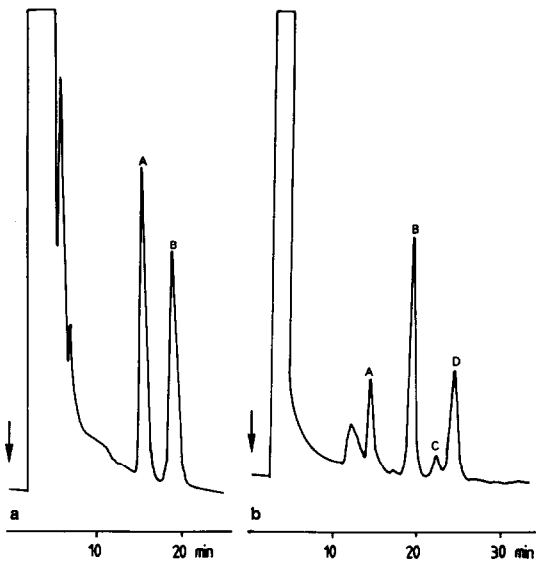

Fig. 1. Chromatograms of (a) human plasma spiked with 100 ng of vincristine (peak A) and 100 ng of vinblastine (peak B) and (b) an extract of mouse plasma, sampled 1 h after injection of 2 mg/kg body weight vinblastine (peak B) and spiked with 100 ng of vincristine as internal standard (peak A). Extra peaks (C and D) after the vinblastine peak are observed.

RESULTS AND DISCUSSION

Chromatography

Fig. 1 shows chromatograms of (a) human plasma spiked with 100 ng of VLB (peak B) (retention time 19.14 min) and 100 ng of VCR (peak A) (retention time 15.60 min) and (b) an extract of mouse plasma, 1 h after injection of 2 mg/kg body weight VLB, and spiked with 100 ng of VCR as internal standard. Some extra peaks can be seen after the peak of VLB: one at 22.10 (peak C) and one at 24.05 min (peak D). These probably represent metabolites, still unknown for the moment. We have never observed similar peaks

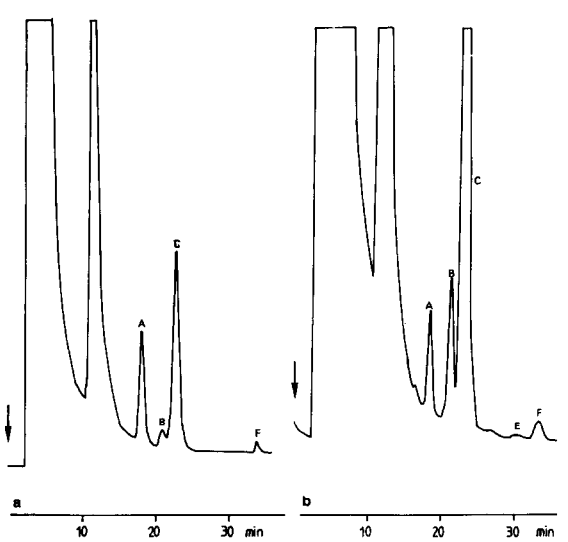

Fig. 2.

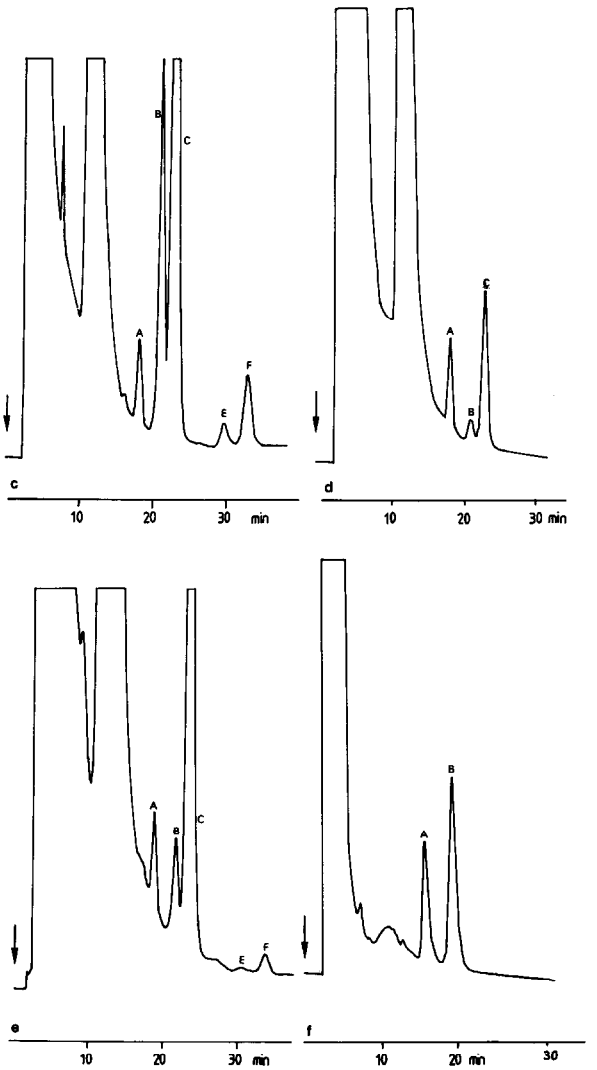

Fig. 2. Chromatograms of tissue samples taken 1 h after injection of vinblastine (2 mg/kg body weight): (a) muscle; (b) kidney; (c) liver; (d) lung; (e) heart; (f) tumour. Peaks: A = vincristine (internal standard, 100 ng); B = vinblastine; C, D, E, and F are probably metabolites (see text).

TABLE I

EXTRACTION RECOVERY OF VLB AND VCR FROM DIFFERENT MOUSE TISSUES AND MO_4 TUMOUR

Detection limit, 10 ng/g of tissue.

Tissue	Mass of lyophilized tissue sample (mg)	Extraction recovery of vinblastine (%)	Extraction recovery of vincristine (%)
Concentration 5 μg/ml			
Tumour	50	60.0 ± 3.5	66.8 ± 3.3
Liver	50	66.2 ± 5.3	62.7 ± 1.5
Kidney	25	63.7 ± 4.5	67.8 ± 4.3
Heart	25	45.0 ± 6.5	58.3 ± 4.5
Lung	30	53.5 ± 5.2	58.0 ± 7.8
Muscle	40	55.2 ± 1.3	64.3 ± 5.3
Concentration 50 μg/ml			
Tumour	50	61.2 ± 2.8	67.8 ± 3.1
Liver	50	67.8 ± 3.5	63.4 ± 2.0
Kidney	25	64.2 ± 3.8	69.2 ± 3.5
Heart	25	48.7 ± 4.7	59.2 ± 3.9
Lung	30	57.5 ± 3.9	61.4 ± 4.8
Muscle	40	56.5 ± 2.1	64.9 ± 5.1
Concentration 500 μg/ml			
Tumour	50	64.2 ± 2.1	66.8 ± 2.9
Liver	50	66.1 ± 2.8	62.7 ± 1.7
Kidney	25	66.0 ± 3.5	67.8 ± 3.8
Heart	25	49.5 ± 4.8	58.3 ± 2.9
Lung	30	53.5 ± 3.7	58.0 ± 4.1
Muscle	40	58.6 ± 2.2	64.3 ± 3.1

in the plasma of VLB-treated humans, and these peaks do not represent deacetylvinblastine, one of the known metabolites, which has a retention time between VCR and VLB [13]. These metabolites or degradation products may be specific for mice, or they may appear in undetectable amounts in human plasma.

Fig. 2 shows the extracts, spiked with VCR (100 ng) as internal standard of different tissues of treated mice, sampled 1 h after injection of 2 mg/kg body weight VLB. Again some extra peaks are observed: peaks C and F in muscle tissue, peak C in heart tissue and peaks C, E and F in kidney, liver and lung tissue, but none of them

TABLE II

EQUATIONS OF CALIBRATION CURVES FOR THE ANALYSIS OF VINBLASTINE IN DIFFERENT TISSUES

Overall curves of the concentration range 0–16 μg/g of tissue; $n = 6$; detection limit, 10 ng/g of tissue.

Tissue	Curve fit	Correlation (r^2)
Tumour	$y = -0.0241(\pm 0.005) + 0.0016(\pm 0.0002)x$	0.9958
Liver	$y = -0.0138(\pm 0.002) + 0.0018(\pm 0.0002)x$	0.9964
Kidney	$y = -0.0037(\pm 0.001) + 0.0018(\pm 0.0001)x$	0.9966
Heart	$y = -0.0321(\pm 0.004) + 0.0017(\pm 0.0002)x$	0.9940
Lung	$y = -0.0941(\pm 0.011) + 0.0016(\pm 0.0002)x$	0.9958
Muscle	$y = -0.0404(\pm 0.007) + 0.0012(\pm 0.0001)x$	0.9824

in tumour tissue. Peak C was mostly found in every sample of normal tissue. Some normal tissue chromatograms show a peak (data not shown) preceding the VLB peak, which is probably deacetylvinblastine.

The detection limit of the system is 10 ng/g of tissue and 1 ng/ml of plasma, using at least 25 mg of tissue or 1 ml of plasma.

Extraction recovery

Table I shows the results of the extraction from the different tissues, including tumour. The recovery of VLB at the lowest concentration varied from 45 to 66.2% with coefficients of variation (C.V.) between 1.3 and 6.5%. The extraction of VCR at this concentration was 58.0–67.8% with a C.V. of 1.5–7.89%. At higher concentrations (100 μl of a 50 or 500 μg/ml solution added to the tissue specimen), the recovery was in the same range (see Table I). The within-day C.V. was 5.1–9.3%. The calibration curve parameters of the drugs extracted from tissue are shown in Table II.

The extraction of both vinca alkaloids from heart tissue showed the lowest recovery. The results from tumour tissue did not differ from these from muscle, where the tumour was implanted.

This combination of lyophilization and chemical extraction makes it possible to use previously described methods of HPLC. The extraction recovery is lower than from physiologial fluids, but still allows the determination of levels of 10 ng/g of tissue. About 30–50% of the drugs are not recovered during the lyophilization, but as the internal standard behaves in the same way as the compounds to be determined, this loss is not a major problem. The technique seems to be suitable for the determination of vinblastine in an animal *in vivo* model, as can be seen from following preliminary studies.

Preliminary pharmacokinetics in plasma and tissues

The time–concentration curves of the disposition of VLB in the different organs are shown in Fig. 3: (A) in all samples organs and in plasma during the first 30 min; (B) for tumour tissues; (C) for muscle tissue. VLB levels are detectable until 3 h in heart, until 6 h in plasma, muscle and liver, until 12 h in kidney and lung and in tumour tissue until 24 h after intravenous injection. The highest concentration can be found in kidney, lung, heart and liver. There was no substantial difference in VLB concentrations in tissues be-

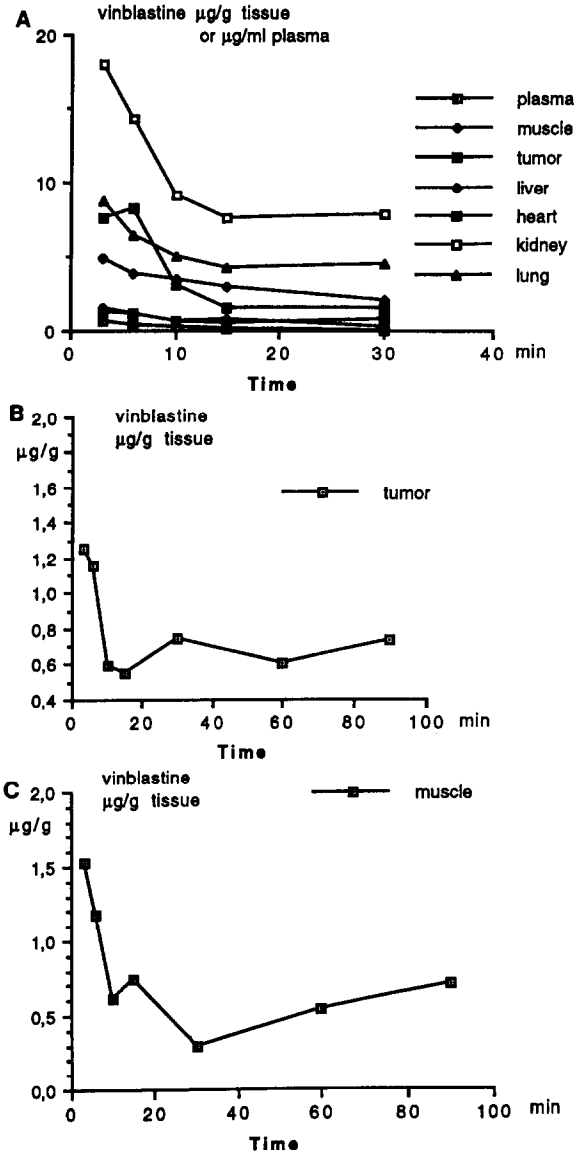

Fig. 3. Time–concentration curves of the disposition of vinblastine in mouse tissues: (A) data for all organs and plasma during the first 30 min; (B) tumour disposition curve; (C) vinblastine concentration in the opposite thigh muscle, both during the first 90 min.

TABLE III

PLASMA LEVELS AND TISSUE DISPOSITION OF VINBLASTINE IN MO_4 TUMOUR-BEARING MICE

Injection of 2 mg/kg vinblastine (mean value of four to seven samples); detection limit, 1 ng/ml of plasma or 10 ng/g of tissue.

Time (min)	Plasma (μg/ml)	Muscle (μg/g)	Tumour (μg/g)	Liver (μg/g)	Heart (μg/g)	Kidney (μg/g)	Lung (μg/g)
3	0.650 ± 0.09	1.530 ± 0.22	1.250 ± 1.23	4.880 ± 0.78	7.520 ± 3.39	17.930 ± 4.09	8.700 ± 1.77
6	0.440 ± 0.20	1.180 ± 0.44	1.160 ± 1.19	3.880 ± 0.91	8.250 ± 4.58	14.261 ± 2.61	6.430 ± 2.05
10	0.250 ± 0.12	0.620 ± 0.15	0.590 ± 0.54	3.510 ± 1.30	3.060 ± 1.12	9.040 ± 1.28	5.020 ± 0.83
15	0.170 ± 0.01	0.750 ± 0.30	0.550 ± 0.30	2.990 ± 0.64	1.580 ± 0.53	7.530 ± 1.23	4.220 ± 0.67
30	0.110 ± 0.02	0.300 ± 0.26	0.750 ± 0.33	2.100 ± 0.99	1.590 ± 0.32	7.830 ± 1.30	4.440 ± 0.53
60	0.090 ± 0.03	0.540 ± 0.10	0.610 ± 0.21	2.590 ± 0.57	0.640 ± 0.07	6.740 ± 1.26	3.100 ± 0.42
90	0.050 ± 0.02	0.710 ± 0.19	0.730 ± 0.17	1.630 ± 0.42	0.920 ± 0.35	9.520 ± 3.38	4.310 ± 1.12
360	0.020 ± 0.01	0.100 ± 0.01	0.290 ± 0.09	0.210 ± 0.04	0	1.590 ± 0.38	0.840 ± 0.30
720	0	0	0.250 ± 0.10	0	0	0.500 ± 0.13	0.410 ± 0.05
1440	0	0	0.190 ± 0.04	0	0	0	0

tween tumour-bearing mice and non-tumour-bearing mice. Note that in tumour tissue the level after 24 h is comparable with the circulating plasma level obtained 10–15 min after injection. All data are listed in Table III.

As mentioned before, extra peaks were seen in the chromatograms of tissue samples. The highest concentrations of these extra peaks were found in samples from liver tissue which could mean that these probable metabolites are excreted in the bile.

CONCLUSION

An HPLC method was developed for the determination of vinca alkaloids in tissues. The method involves ion-pair extraction and selective reversed-phase HPLC, based on a cyanopropyl stationary phase. Lyophilization of tissues provided adequate recovery of the drugs of interest. The technique was applied to the determination of VLB in mice tissue samples and implanted tumours.

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