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Determination of vinblastine in tumour tissue with liquid chromatography–high resolution mass spectrometry

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ABSTRACT

There are virtually no analytical methods that describe determination of vinblastine and other vinca alkaloids in tumour tissue, albeit quantitative data on tumour drug amount is essential for maximal benefit of a particular anticancer treatment. The analytical method presented herein uses state-of-the-art sample preparation, separation and detection techniques to allow sensitive and selective determination of vinblastine in tumour tissue. After cryogenic grinding and sonication, tumour suspensions were extracted by Oasis MAX solid phase extraction and analysed for vinblastine with ultra-high performance liquid chromatography coupled to positive electrospray ionisation–high resolution mass spectrometric detection. The developed analytical method quantifies vinblastine down to 23 ng/g tumour tissue and shows satisfactory linearity ($r^2 > 0.99$), precision (1.1–8.2%), accuracy (98%) and high selectivity with almost complete absence of matrix effects. The proposed method was found suitable to follow vinblastine levels in mice tumours and could be used to support preclinical pharmacologic studies.

1. Introduction

Vinca alkaloids are prescribed for wide variety of cancers including non-small cell lung cancer, breast cancer, bladder cancer, lymphomas and leukaemia [1]. Vinblastine (VBL) and vincristine (VCR) are naturally occurring alkaloids derived from *Catharanthus roseus* plant, but several semi-synthetic derivatives and metabolites, including vinorelbine, vinflunine, vindesine and 4-O-deacetylvinorelbine, exhibit similar pharmacological activity [1]. The mechanism of action of vinca alkaloids is binding to beta-tubulin and disruption of microtubule function during mitosis, which in turn leads to mitotic arrest and cell death [1–3]. There is a great deal of interest into the pharmacokinetic properties of these drugs, even though it can be noticed that VBL has received less attention as compared to other vinca alkaloids [1]. To support pharmacokinetic and pharmacologic investigations the knowledge on drug levels in a target tissue is of crucial importance, therefore fast, sensitive, selective and reliable bioanalytical methods are essential.

Bioanalytical quantitative methods consist of two steps, sample preparation and analysis, that both impact the accuracy, precision, selectivity and sensitivity of the analytical methods [1]. Sample preparation is an important segment of quantitative bioanalysis, particularly when followed by liquid chromatography coupled to mass spectrometric detection (LC–MS). Since in the trace analysis the amount of matrix components that can potentially interfere with quantification or identification of a target analyte is relatively higher, this creates higher demands on the selectivity and sensitivity of the applied analytical method. During sample preparation interfering matrix compounds such as proteins, salts and lipids are removed from the sample. Failure to effectively remove these matrix interferences would result in a suppression of ionisation efficiency of the electrospray source and hence lower sensitivity. Sample preparation methods differ according to their selectivity for a given analyte and ability to efficiently remove matrix interferences. Most bioanalytical sample preparation methods are still limited to only protein precipitation or liquid–liquid extraction. This way VBL has been determined in mouse fibrosarcoma cells [4], plasma [5–7] and solid tissue samples [7]. In comparison to protein precipitation or liquid–liquid extraction

Abbreviations: APCI, atmospheric pressure chemical ionisation; FWHM, full width at half maximum; HRMS, high resolution mass spectrometry; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; ME, matrix effect; MS, mass spectrometry; QTOF MS, quadrupole time-of-flight mass spectrometer; RE, recovery; RS, reconstitution solvent.; SPE, solid phase extraction; VBL, vinblastine; VCR, vincristine; WS, wash solvent.

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solid phase extraction (SPE) is a more advanced sample preparation method that offers an increased selectivity and less laborious sample clean-up [8], but it has only rarely been employed for extraction of VBL. The two examples include reversed phase SPE from plasma and urine [8,9]. Alternative sorbents to reversed phase SPE have been developed, such as ion exchange SPE sorbents and molecularly imprinted polymers. Ion exchange SPE allows an extraction of ionic species from a sample, assuming that ion strength and pH are carefully controlled. This way most matrix interferences are removed from the extract [10]. To the best of our knowledge, this is the first study to have employed the ion exchange retention mechanism for isolation of VBL from biological samples.

Before the year 2000 the analytical methods for determination of VBL in biological matrices mainly involved LC coupled to fluorescence [5,7], ultraviolet [11] or electrochemical detection [8]. Nowadays, however, LC–MS has become the method of choice for quantification of VBL and vinca alkaloids in general. The main reason is that the sensitivity and selectivity of the LC–MS are higher as compared to other analytical methods, which makes LC–MS the method of choice in therapeutic drug monitoring and drug metabolism studies, as well as drug development process [9]. However, only Ramirez et al. [6] and Achanta et al. [9] employed LC–MS/MS for quantitation of VBL, eventhough the compound has commonly been used as an internal standard for determination of other vinca alkaloids [12–16]. Both groups [6,9] report on using an atmospheric pressure chemical ionisation source (APCI), whereas the behaviour of VBL under electrospray ionisation (ESI) conditions is yet to be discussed.

Based on the gaps identified in Introduction and in order to follow the amount of VBL in tumour tissue, our aim was to develop a selective and sensitive analytical method for determination of VBL, which is based on a sample preparation with an ion exchange SPE, and analysis with LC coupled to positive electrospray ionisation and detection with high resolution MS (LC–ESI(+)–HRMS).

2. Materials and methods

2.1. Chemicals and reagents

Sulphate salts of VBL and VCR were purchased at Carbone Scientific Co. (London, UK) and were of 98% purity. VBL for injection 10 mg/10 mL vial (vinblastine sulphate) was obtained from Pharmachemie B. V. (Haarlem, Netherlands). LC–MS grade acetonitrile and water were purchased at J.T. Baker (Phillipsburg, NJ, USA), whereas ammonium formate ($\geq$99.0% purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All the remaining solvents and chemicals (methanol, water, hydrochloric acid, sodium acetate, formic acid and triethylamine) were of analytical grade purity. Solid phase extraction sorbents tested were: Oasis MAX 60 mg/3 mL (Waters Corp., Milford, MA, USA), Strata XCW 60 mg/3 mL (Phenomenex, Torrance, CA, USA), Oasis WCX 60 mg/3 mL (Waters Corp.), Evolute ABN 50 mg/3 mL (Biotage AB, Uppsala, Sweden) and Oasis HLB 60 mg/3 mL (Waters Corp.).

2.2. Animals and tumours

Male and female A/J mice aged 6–8 weeks were obtained from Institute of Pathology, Faculty of Medicine, University of Ljubljana, Slovenia and Harlan, Udine, Italy. Mice were housed under specific pathogen-free conditions at a constant room temperature and humidity with a 12 h light/dark cycle. Food and water were provided *ad libitum*. Prior to the experiments the animals were subjected to an adaptation period of 2 weeks. All procedures on animals were performed in accordance with the guidelines for animal experiments of the EU directives and the permission from the Veterinary Administration of Ministry of Agriculture and the Environment of the Republic of Slovenia (Permission No. 34401-3/2012/2 and 34401-12/2009/6). The average body weight $\pm$ SD of mice was 26 $\pm$ 1.3 g. Solid tumours were induced in the back of mice by s.c. injection of 5×10^5 SA-1 murine fibrosarcoma cells resuspended in 0.1 mL of physiological solution. Cells for tumour induction were obtained from the tumour grown intraperitoneally as ascites in donor mouse. After VBL treatment (see Section 3.4 Method application) tumours were weighed and shock-frozen in liquid nitrogen and stored at -80 °C until analysis.

2.3. Preparation of stock and working solutions

VBL and VCR (internal standard) stock solutions with concentrations of 200 μg/mL were prepared in methanol. VBL stock solution was diluted by serial dilution with a reconstitution solvent (10 mM NH_4HCOO in LC–MS water mixed with 10 mM NH_4HCOO in acetonitrile; ratio 9/1) to obtain a series of working standards in concentration range of 0.005–1 μg/mL. Calibration standards were prepared by spiking tumour suspensions with corresponding amounts of VBL that give the same final concentrations as the working standards. VCR stock solution was diluted in the reconstitution solvent to give the concentration of 1 μg/mL, where 20 μL of this solution was added into samples as the internal standard.

2.4. Sample preparation

Frozen tumours were grinded to fine powder with liquid nitrogen using a mortar and a pestle and transferred to a 15 mL centrifuge tube using a pre-chilled spatula. The residual sample was collected by washing the mortar and pestle surfaces with 14 mL (divided into four aliquots) of deionised water acidified to pH 2 with hydrochloric acid. After addition of 20 μL of 1 μg/mL VCR solution, the suspension was sonicated for 1 h at 35 kHz in an ultrasonic bath (VWR International, Radnor, PA, USA) and then centrifuged at 8600*g* for 20 min. The supernatant was filtered through 0.45 μm Minisart® NML (Sartorius, Göttingen, Germany) surfactant-free cellulose acetate syringe filters and loaded onto the SPE cartridge, which was preconditioned with 3 mL methanol and equilibrated with 3 mL deionised water acidified to pH 2. The pellet and surfaces of the centrifuge tube were washed with 3 × 1 mL of pH 2 deionised water, filtered and then loaded on the sorbent. The sorbent was subsequently washed with 6 mL of 10% methanol in deionised water, followed by 6 mL of 10% methanol in 50 mM CH_3COONa. The sorbent was dried with a vacuum pump for 30 min; then VBL was eluted with 3 × 2 mL 2% HCOOH in methanol. The eluate was dried under a nitrogen stream and the dry extract was kept at 4 °C until the analysis (less than 2 weeks). Before the analysis the extracts were reconstituted with 150 μL of 10 mM NH_4HCOO in LC–MS water mixed with 10 mM NH_4HCOO in acetonitrile in ratio 9/1 (RS).

2.5. LC–MS operational parameters

Quantitative analysis was performed with Waters Acquity Ultra-performance liquid chromatograph (UPLC, Waters Corp., Milford, MA, USA) coupled to a Waters Premier hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (QTOF MS). The UPLC was equipped with a binary solvent delivery system and an autosampler. The injection volume was 7.5 μL. Separation was achieved at 50 °C by using a 5-cm-long Acquity UPLC™ BEH Shield C-18 (Waters Corp.) column with 1.7 μm particle size and 2.1 mm internal diameter. VBL and VCR were analysed under electrospray ionisation in positive mode (ESI(+)). The mobile phases were (A) water and (B) acetonitrile, both mixed with

10 mM ammonium formate. The gradient started with 10% B which was increased to 90% in 5 min, decreased back to 10% in 0.5 min and kept so until 7 min. The flow rate was 0.3 mL/min. The UPLC system was coupled to QTOF MS equipped with an electrospray ionisation interface. The capillary voltage was set to 3.0 kV, while the sampling cone voltage was 45 V. Source and desolvation temperatures were set to 130 °C and 300 °C, respectively. The nitrogen desolvation gas flow rate was 530 L h^{-1}. The first quadrupole was operated in rf-only mode, while detection was performed in the TOF mass analyser. MS data were acquired over an m/z range of 800–900 at a collision energy of 4 V. Data were collected in a centroid mode, with a scan accumulation time set to 0.25 s and an interscan delay of 0.02 s. The data station operating software was MassLynx v4.1. Prior to analysis, the instrument was calibrated over a mass range 50–1000, using a sodium formate calibration solution. Reproducible and accurate mass measurements at a mass resolution of 10,000 FWHM were obtained using an electrospray dual sprayer with leucine enkephalin ($[M+H]^+$ 554.2615, $[M-H]^-$ 556.2271) as the reference compound. The latter was introduced into the mass spectrometer alternating with the sample via a Waters Lock Spray device. For VBL its protonated molecule at 811.4282 ± 5 ppm and for VCR the $[M+H]^+$ at 825.4075 ± 5 ppm were followed. To acquire VBL MS/MS spectra collision energies between 15 V and 30 V were applied.

2.6. Validation procedures

Validation of the analytical method included the assessment of carryover, recovery, SPE efficiency, matrix effect, selectivity, sensitivity, linearity, precision and accuracy. Validation procedures were performed according to Matuszewski et al. [17], Yin et al. [18] and Knoll [19]. Due to ethical reasons the number of tumour replicates applied in the validation study was minimised to the lowest required level that still allows a reliable validation.

For the assessment of carryover we considered two possible sources, i.e. the LC injection and the sample preparation. The carryover of the LC–MS analysis was determined by injecting a blank plasma sample following the injection of highest concentration analytical standard (1 μg/mL). Carryover of the sample preparation was tested by grinding a blank tumour (tumour without treatment) with the same mortar and pestle as a tumour treated with highest dosage of VBL, which was grinded beforehand.

For determination of the matrix effect (*ME*) matrix-matched and solvent-based standard calibration curves were drawn and the corresponding slopes in matrix and in solvent were compared. Matrix effect is defined as: $ME\ \% = (slope\ in\ matrix / slope\ in\ solvent) \times 100$. A value of 100% indicates the absence of the matrix effect. There is signal enhancement if the value is > 100% and signal suppression if the value is < 100% [18]. According to Matuszewski [17] the matrix effect is determined as a percentage of VBL peak area spiked after extraction (B) divided by VBL peak area spiked into the reconstitution solvent (A): $ME\ \% = B/A \times 100$. The matrix effect was calculated according to both described methods.

SPE efficiency (*SPE*) was determined in three replicates as a percentage of VBL peak area spiked into a blank tumour suspension (C) and treated as described in Section 2.4 Sample preparation versus corresponding VBL peak area spiked after extraction into a dry sample extract of a blank tumour (B): $SPE\ \% = C/B \times 100$.

Recovery (*RE*) was determined in three replicates as a percentage of VBL peak area spiked into a blank tumour suspension (C) and treated as described in Section 2.4 Sample preparation versus VBL peak area spiked in the same concentration into the reconstitution solvent (A): $RE\ \% = C/A \times 100$.

Selectivity of the method was ascertained by high resolution mass spectrometry, where the accurate masses of VBL and VCR were followed at ± 5 ppm tolerance interval. The method selectivity was further confirmed by analysing extracts obtained from five SA-1 tumours, which were not treated with VBL. Endogenous peaks were not observed at the retention time of VBL ± 1 min in any of the tumour extracts analysed. Selectivity of the sample preparation method is also discussed in Section 3.1 Development of the sample preparation method.

Sensitivity is expressed as a limit of detection (LOD) and limit of quantification (LOQ). LOD is calculated as the analyte concentration that produces a chromatographic peak having a height equal to three times the standard deviation of the baseline noise [19]. LOQ is calculated as 3.33 × LOD.

Linearity of the standard curve was determined by plotting the peak area ratio of VBL to VCR versus VBL concentration. The sample concentrations were calculated from equation determined by linear regression of the standard matrix-matched curve.

The assessment of the precision involved estimating interday and intraday repeatability of the injection, and repeatability of the analytical method. The interday repeatability of the injection was assessed by performing duplicate injections of VBL standard over 3 days. The intraday repeatability was determined as the CV % of three injections of VBL standards within 1 day. The analytical method repeatability was determined by analysis of three replicate samples prepared as described in Section 2.4 Sample preparation.

Accuracy of the method was expressed by comparing the difference between the measured and the spiked concentration: $accuracy\ error\ \% = [(mean\ observed\ concentration - mean\ spiked\ concentration)/spiked\ concentration] \times 100$ [20].

3. Results and discussion

3.1. Development of the sample preparation method

Effective disruption of biological samples is an important step in sample preparation that involves one or a combination of processes aiming to liberate analyte molecules in a homogenised sample. There exist a number of methods used for sample disruption [21], among which cryogenic grinding with mortar and pestle is a widely used method, since it is effective, simple and requires a relatively inexpensive apparatus. Yet, a significant problem is that small samples can be essentially lost when ground into the surface of the mortar, which leads to poor analyte recovery. Since there exists no standard reference material for VBL in a tumour tissue, it is practically impossible to assess the losses of the analyte during the cryogenic grinding process. However, since we proved there was no carryover when subsequently grinding a tumour containing highest concentration of VBL followed by a blank tumour (see Section 3.3 Analytical method validation), we believe that by careful wash of the residuals from mortar and pestle surfaces we managed to quantitatively transfer the analyte into the receiving centrifuge vessel.

Sonication is a homogenisation method that uses shock waves thus disrupting a sample by pressure. It is a powerful method, if samples contain small particles, such as cells or homogenised tissue, but for solid tissue like tumour, standalone sonication is a very poor method. However, a very effective sample disruption typically uses two or more homogenisation processes of which a combination of cryogrinding as a first step and sonication as the second appears to be of uttermost efficiency [21]. The combination of these two homogenisation processes was employed to liberate VBL from tumour cells into “wash and sonication solvent” (WS solvent).

The ground of WS solvent selection is its compatibility with a subsequent mixed mode solid phase extraction. We compared performance of three WS solvents, including deionised water, 100 mM sodium acetate and water acidified to pH 2 with a concentrated HCl. From Fig. 1 it is evident that addition of acid

results in improved VBL recovery as compared to the addition of salt; further the tumour suspension in the acid is after sonication visibly clearer than the one in the salt. Therefore, the deionised water acidified with HCl to pH 2 was selected as the WS solvent. Fig. 1 further shows the process of optimisation of sorbent wash phase 1, which is discussed further on in the article.

In comparison to conventional sample preparation methods such as liquid–liquid extraction or protein precipitation, SPE is more selective thus giving cleaner extracts with less matrix interferences responsible for a suppression of analyte ions in the subsequent LC–MS analysis. For extraction of vinca alkaloids mostly a reversed phase SPE was employed [9,13,14,22,23], except for the study by Embree et al. [24], where VCR was extracted by a cation exchange sorbent.

VBL is a relatively large molecule with an elemental composition of $C_{46}H_{58}N_4O_9$ giving a molecular mass of 810.4204 that has a log K_{ow} 3.7. The molecule (Fig. 2) involves several highly polarised functional groups, including basic amines and several hydroxyl moieties. Its dissociation constants at 5.0 and 7.4 [25] suggest that the amine groups will be partially protonated at neutral and acidic pH. VBL should thus exhibit reversed phase and cation–anion interactions with an SPE sorbent.

In the present study we compared % recovery of VBL on different SPE sorbents including two reversed phase (Evolute ABN and Oasis HLB), two mixed mode–cation exchange (Strata XCW and Oasis WCX) and a mixed mode–anion exchange sorbent (Oasis MAX). The cartridges were initially subjected to general producer-recommended extraction procedures. As evident from Fig. 3 both reversed phase sorbents showed relatively poor recovery, whereas there is an apparent difference between the two mixed mode–cation exchange sorbents, with Oasis WCX yielding higher recovery. Yet, despite neither VBL nor VCR have the potential for an anion exchange, Oasis MAX gives superior performance for both compounds. It should be however emphasised that the highest recovery does not necessarily mean highest SPE efficiency, but rather highest selectivity for a given analyte. This is justified by the fact that the reversed phase sorbents show poorest recovery, which may be due to coextracted matrix interferences that suppress the analyte ions when ionised by ESI(+). In the mixed mode–cation exchange sorbent VBL and VCR can be retained by both reversed phase and cation exchange interactions, whereas in the mixed mode–anion exchange sorbent only the reversed phase interactions are relevant for both compounds. Thus, despite having lower retention capacity, the recovery is higher in case of Oasis MAX because more matrix interferences are being washed off by applying carefully the designed sorbent wash procedure.

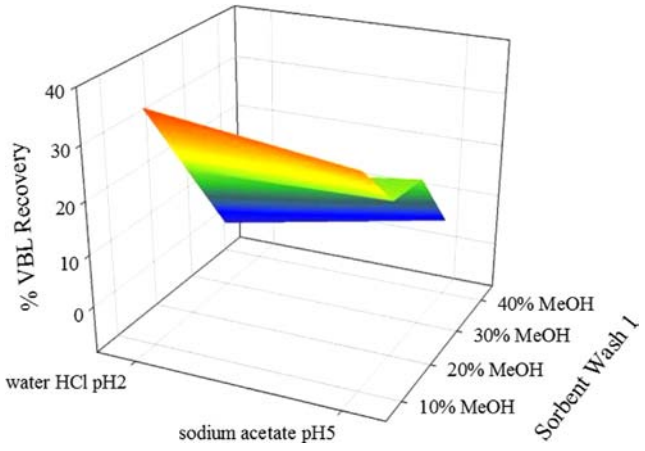

Fig. 1. VBL% recovery with respect to the WS solvent and sorbent wash 1.

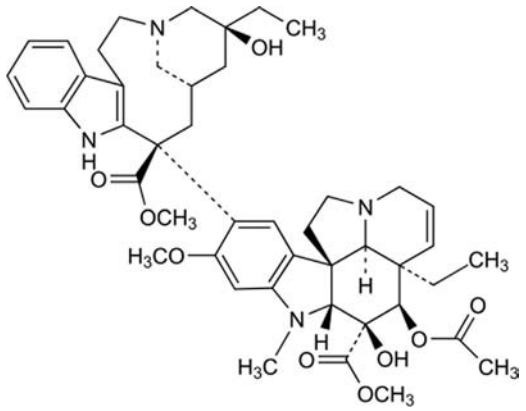

Fig. 2. Chemical structure of VBL.

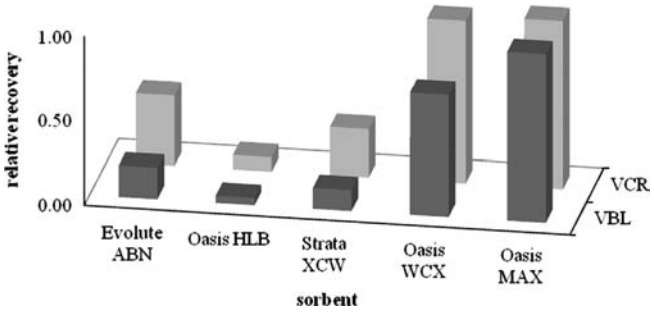

Fig. 3. Selection of most appropriate sorbent. VBL and VCR.

Sorbent wash is one of the crucial steps in the SPE, especially when complex biological matrices are processed. By testing 0–40% (v/v) methanol we observed optimum recovery at 10% methanol in deionised water (Fig. 1). By adding the second wash step, where 10% methanol dissolved in 50 mM sodium acetate is applied, the recovery of VBL is further improved.

The choice of an elution solvent is again based on the strength and type of interactions between an analyte and elution solvent. Methanol showed best recovery among pure organic elution solvents, where an addition of 2% formic acid improved the recovery for VBL and VCR by 50% and 20%, respectively.

3.2. LC–MS analysis

VCR and VBL were easily ionised by the ESI(+) technique. Depending on the mobile phase tested we observed considerable variations in VBL and VCR peak shapes, their ionisation yields, formation of alkali adducts and multiple charged species. In order to achieve most favourable ionisation of VBL we modified aqueous and organic mobile phases with HCOOH and ammonium formate in different concentrations. Best chromatographic response was achieved by adding 10 mM ammonium formate into both aqueous and acetonitrile mobile phase. Under optimised mobile phase gradient (see Section 2.5 LC–MS operational parameters) VCR and VBL eluted at 2.7 min and 2.9 min, respectively (see chromatogram in Fig. 4(a)). Best sensitivity in ESI ionisation is normally achieved at cone voltages between 20 V and 30 V. Yet, for VBL these voltages caused formation of a double charged molecular ion at 406.2, which was under such conditions 2.8–5-fold more abundant than the single charged protonated molecule at 811.4 (Fig. 4(b)). Such dispersion of charges during ionisation is undesirable, since it results in an overall sensitivity reduction. By increasing the cone voltage up to 50 V the ratio single charged versus double charged ion changed in favour of the first, where best response was shown at 45 V. Finally, by replacing formic acid with ammonium formate along by applying high sampling cone

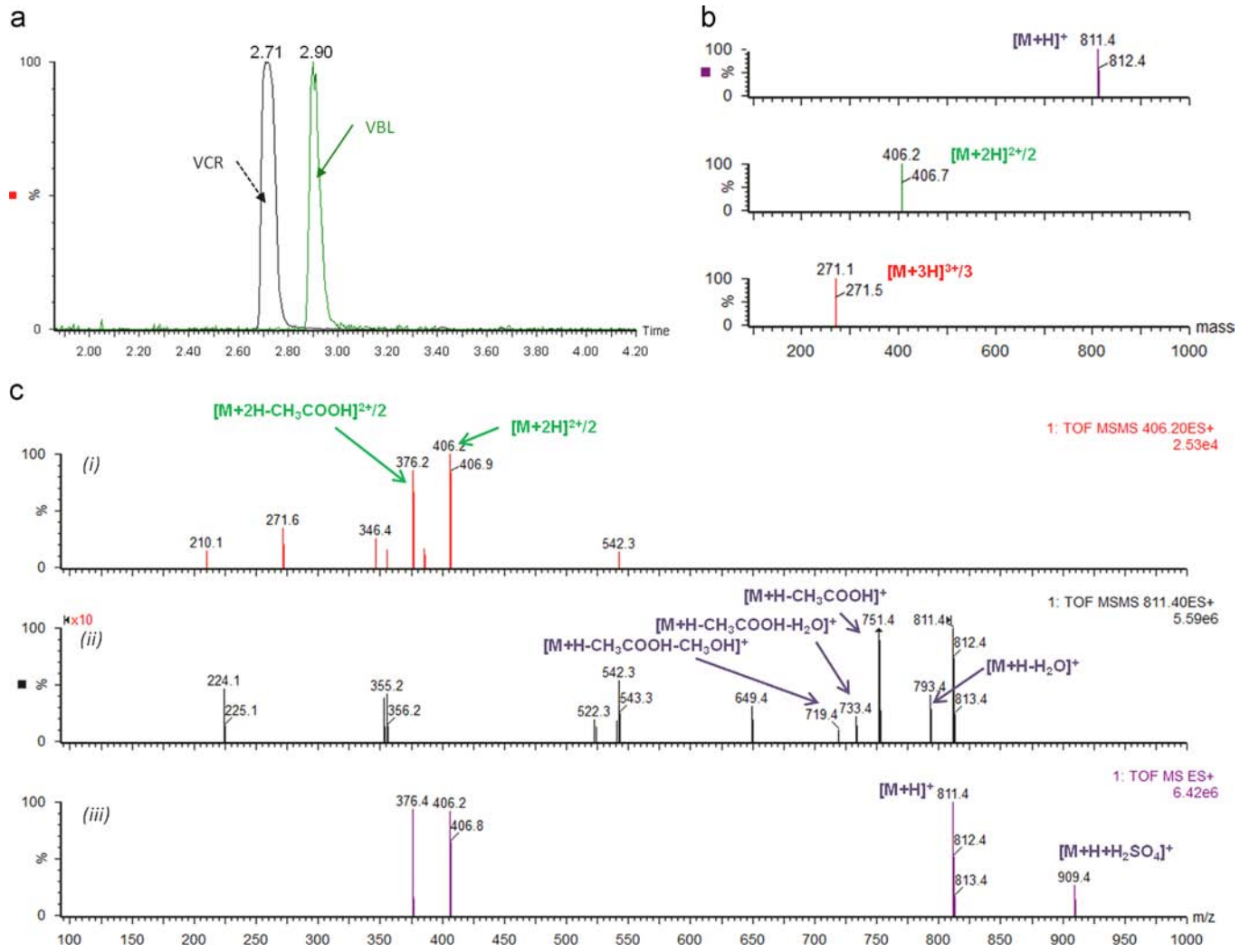

Fig. 4. (a) Separation of VCR and VBL; (b) calculated single-, double- and triple-charged VBL molecular ions; and (c) actual VBL MS and MS/MS spectra: (i) MS/MS 406.2, (ii) MS/MS 811.4, and (iii) TOF MS of VBL.

voltage we managed to eliminate the formation of the double charged molecular ion, which considerably improved the LC–MS method sensitivity.

In Fig. 4(b) we further propose a triple charged molecular ion at 271.1, which was not detected in VBL mass spectra. However, in Fig. 4(c-i) a 0.5 Da higher mass at 271.6 *m*/*z* is evident, which happens to be a fragment ion of the double charged VBL species. Fig. 4(c) shows the comparison of MS/MS spectra of the double charged VBL ion at 406.2, single charged protonated molecule at 811.4 and TOF MS spectrum of the VBL. The most apparent ion obtained by fragmentation of the double charged molecular ion 406.2 is 376 *m*/*z* (Fig. 4(c-i)), which corresponds to 751 *m*/*z* in the MS/MS spectrum of the protonated molecule $[M+H]^+$ 811.4 (Fig. 4 (c-ii)), and was formed by cleavage of acetic acid. We also observed neutral losses of two water molecules and a methanol molecule (Fig. 4(c-ii)), and addition of sulphate in the TOF MS mode (Fig. 4 (c-iii)). All fragments and adducts were resolved by employing HRMS at mass errors lower than 3.4 ppm. We were unfortunately unable to recognise few lower-mass ion fragments due to the complexity of VBL chemical structure.

3.3. Analytical method validation

Carryover causes the appearance of an analyte in a run when a blank is injected. The peaks attributed to carryover in LC runs generally originate from one of three sources: general laboratory contamination, sample preparation or the autosampler [26], though the majority of bioanalytical studies consider only the possibility of the autosampler carryover. As evident in Table 1, the carryover of 0.34% was determined in case no additional flushing of the needle was performed. However, taking into account that the highest concentration of VBL measured in a tumour sample was 74 ng/mL, the concentration of VBL left over in the subsequently injected sample is $<$LOD, which makes such carryover acceptable for a given application. Even so, to diminish the possibility of carryover the sample sequence was arranged from lowest to highest predicted VBL concentration. In addition, solvent blanks were injected in the sequence of one blank per five samples and were checked for the absence of VBL, whereas an additional needle flushing was performed as needed, or at least at every start-up and shutdown of the LC system.

Another source of possible carryover is a risk of cross-contamination during sample preparation, which is particularly underlined during cryogenic grinding step. Since tumour samples are grinded using the same mortar and pestle there exists a possibility for VBL residues to adsorb on the surfaces and contaminate the samples grinded subsequently. By proving the absence of VBL in a blank tumour processed after a tumour treated with the highest dosage of VBL, we confirmed that by thoroughly cleaning the grinding apparatus we sufficiently diminished the possibility of carryover.

Table 1
Validation of the analytical method for determination of VBL in tumour tissue.

Validation parameter	
Carryover	Autosampler: 0.34%; sample preparation: no
Matrix effect %	99–107
SPE %	64 ± 5
Recovery %	65 ± 5
Sensitivity: LOD/LOQ (ng/mL)	LOD = 0.3; LOQ = 1.1
Sensitivity: LOD/LOQ (ng/g)	LOD = 6.9; LOQ = 23
Linearity (r^2); conc. range 1–500 ng/mL	0.9994
Repeatability of measurement: intraday %	1.1
Repeatability of measurement: interday, %	8.2
Repeatability of analytical method (three replicates), %	7.5
Accuracy error %	2

The matrix effect might exert a detrimental impact on the method performance parameters including LOD, LOQ, linearity, accuracy, and precision, hence it has to be minimised and evaluated during the validation procedure. Species that may cause ion suppression are endogenic species already present as components of the sample that co-extract with the analyte during sample preparation. The potential ion suppressors are polar compounds (phenols, arylsulfonates) and organic molecules, in particular phospholipids, carbohydrates, peptides and, in general, compounds or metabolites characterised by a chemical structure similar to the target analyte. Coextracted substances present in the injected sample can cause relevant determination problems, especially when they are present at high concentration and co-elute with the analyte, so modifying its signal [27]. The degree of ion suppression mainly depends on the sample preparation method [28], where an increased selectivity of sample preparation, particularly the extraction, decreases the matrix effect. As depicted in Table 1 we achieved the matrix effect of 99–107%, which is within the precision uncertainty. This implies that the sample matrix has practically no effect on determination of VBL, demonstrating a high selectivity of the developed analytical method.

As shown in Table 1 the SPE efficiency is 64%, which is acceptable [18,29], considering that the SPE sorbent is anion exchange. Ideally, the SPE efficiency of 100% should be reached, yet, mixed mode–anion exchange sorbents are characterised with a lower carbon load and require a more demanding method development, making a trade-off for high selectivity achieved with the sample preparation. As a sum of the matrix effect and SPE efficiency the recovery of 65% was calculated. The developed analytical method also shows sufficient linearity, precision and accuracy (Table 1).

The method sensitivity is evaluated by LOD and LOQ, as given in Table 1. Having the LOQ of 1.1 ng/mL or 23 ng/g tissue this analytical method proves to be very sensitive and is the first to determine VBL in tumour tissue by employing advanced sample preparation and detection methods. To the best of our knowledge only one method for determination of VBL in tumour tissue is described in the scientific literature [7], but it is based on detection using an HPLC coupled to a fluorescence spectrophotometer, which in turn results in lower sensitivity and selectivity of the method [7]. Another similar application was developed by De Smet et al. [4], who determined VBL in MO_4 mouse fibrosarcoma cell culture; yet, again they use a less selective detection method (UV) and unfortunately do not report on the method sensitivity [4].

3.4. Method application

The analytical method was successfully applied to quantify the concentrations of VBL in tumour samples of mice that received intraperitoneal injection of 12.5 μg VBL sulphate dissolved in 0.2 ml physiological solution. Mice were consecutively treated every 4 h (on time points 0 h, 4 h, 8 h, and 12 h), meaning that the first group of mice received 12.5 μg of VBL at the time point 0 h, the second group received 12.5 μg of VBL at time point 4 h etc. On every time point of the VBL injection, eight mice were sacrificed 10 min post administration and tumours were excised, weighted (mean ± SD: 77.1 ± 45.3 mg) and analysed for VBL. While confirming the absence of VBL in the control samples, it was shown that after receiving the first two VBL doses its tumour concentrations were comparable (Table 2). A significant, approximately 3-fold increase in VBL tumour concentration was observed after the third intraperitoneal injection of VBL (Table 2).

Table 2
Concentrations of VBL in tumour samples of mice that received intraperitoneal injections of 12.5 μg VBL dissolved in physiological solution at 4 h interval in different schedules (on time points 0 h, 4 h, 8 h, and 12 h).

VBL dosage/time point	VBL concentration (ng/g)	Number of biological replicates
VBL 0 h	114.1 ± 42.1	8
VBL 4 h	124.5 ± 23.4	8
VBL 8 h	398.1 ± 49.5	8
VBL 12 h	347.6 ± 38.0	8

4. Conclusions

The knowledge about the amount of chemotherapeutic drug present in the tumour is of crucial importance when combining different treatment agents or modalities, i.e. either in case of different combined chemotherapeutic schedules or when systemic agents are combined with irradiation. Namely, the timing of application of each individual treatment component should be based on its mechanism of action to achieve a maximal treatment benefit [30,31]. Therefore, the quantitative data about tumour drug amount is essential for optimal scheduling. With this in mind, we developed an analytical method that allows trace-level determination of VBL in mice tumour tissue, using VCR as the internal standard. The method employs cryogenic grinding and sonication followed by mixed mode–anion exchange solid phase extraction as the sample preparation method. For analysis liquid chromatography coupled to high resolution mass spectrometry is used. The method was successfully applied to follow the concentration of VBL in mice tumours treated with different cumulative dosages of VBL. Based on the similarities in physico-chemical properties of VBL and other vinca alkaloids and their metabolites the developed analytical method may potentially serve as a basis for determination of other structurally related compounds in tumour tissue.

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