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Simultaneous quantification of vinblastine and desacetylvinblastine concentrations in canine plasma and urine samples using LC–APCI–MS/MS

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ABSTRACT

A highly sensitive and specific liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC/APCI–MS/MS) method has been developed and validated for simultaneous quantification of vinblastine and its metabolite, desacetylvinblastine, in canine plasma and urine samples. Plasma and urine samples were processed by a solid phase extraction procedure. The optimal chromatographic behavior of these analytes was achieved on pentafluorophenyl (PFP) propyl analytical column (5 μm, 50 × 2.1 mm) under isocratic elution of 0.75 mL/min with a mobile phase of 5 mM ammonium acetate and methanol. The samples were analyzed in positive ion, multiple reaction monitoring mode. The calibration curves were linear over 0.125–2 ng/mL (lower calibration curve); 2–100 ng/mL (higher calibration curve) and 0.125–5 ng/mL for vinblastine and desacetylvinblastine in plasma, and over 1–2000 ng/mL and 0.5–100 ng/mL for vinblastine and desacetylvinblastine in urine samples, respectively. The limits of quantitation of vinblastine and desacetylvinblastine were 0.125 ng/mL in both matrices. The intra and interday accuracy was above 89% and precision below 8.6% for both analytes in both matrices. The developed method was successfully applied to ongoing *in vivo* vinblastine pharmacokinetic studies in dogs.

1. Introduction

Vinblastine (vincaleukoblastine; VLB) is a vinca alkaloid isolated from dicotyledonous Madagascar periwinkle plant, *Catharanthus roseus* (family: Apocyanaceae). Vinblastine was the first vinca alkaloid isolated and has been used for the treatment of soft tissue tumors both in human and veterinary medicine as a single agent or in combination with other anticancer drugs. 4-O-desacetylvinblastine (DVLB) is a metabolite of vinblastine that is itself considered to be a potent and active anticancer xenobiotic [1]. Vinca alkaloids work by binding to beta-tubulin and preventing the formation of microtubules during mitosis, thereby arresting cell division [2–4]. Vinca alkaloids were also shown to inhibit angiogenesis [5]. In order to increase the therapeutic utility or decrease the potential for toxic side effects, semi-synthetic vinca alkaloids, such as vinflunine, vindesine sulfate and vinorelbine (VRB) were developed. Although all of these vinca alkaloids share common features in structure and affinity to microtubules, there is variability in their therapeutic efficiency, therapeutic indications, and toxicities [6]. Currently, toxicity adjusted dosing is the most accepted method of dose adjustment for vinblastine administration in dogs [7,8].

A variety of methods, including radioimmunoassay and high performance liquid chromatography (HPLC) coupled with ultraviolet (UV), fluorescence, or electrochemical detection have been used to quantify vinblastine concentrations in biological matrices [9–12]. A reversed phase – HPLC/fluorescence method was successfully developed in this laboratory for the analysis of *vinca* alkaloids with a maximal sensitivity of 1 ng/mL. Use of the HPLC/fluorescence method to assess preliminary pharmacokinetic samples in canine plasma indicated the need for an assay with even greater sensitivity, with quantitation of vinblastine plasma concentrations well below 1 ng/mL. As species specific differences in blood and urine constituents can result in matrix effects and differences in analyte recovery, assay methods must be validated in the species of interest. In addition, the natural variability found between dog breeds can result in considerable intersubject variability, necessitating the use of an assay with a wide working range in analyte concentrations. To the best of the authors' knowledge, there are no published reports on highly sensitive and specific analytical methods to quantify the concentrations of vinblastine and desacetylvinblastine in canine plasma and urine. The literature indicated successful liquid chromatography tandem mass spectrometry (LC/MS/MS) methods with the required sensitivity [13], so this laboratory began pursuing an LC/MS/MS method for the quantification of

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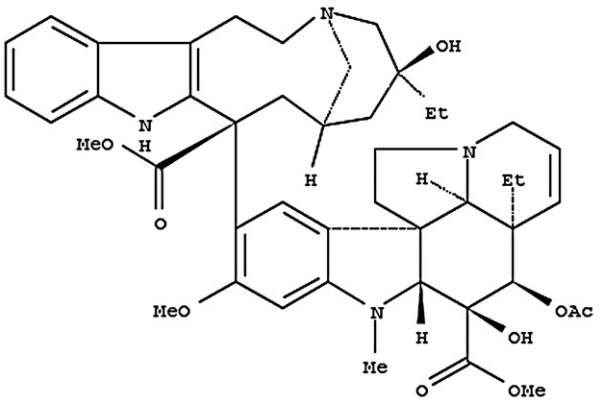
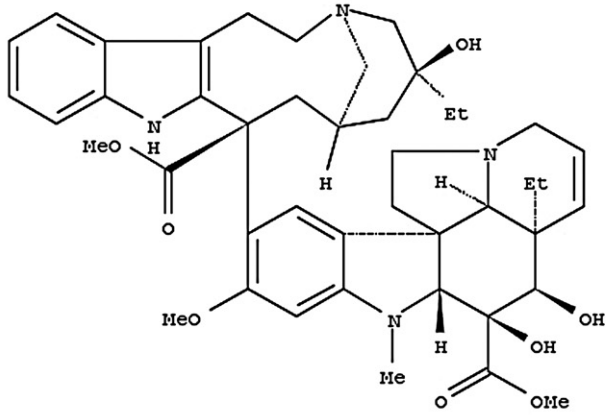
Vinblastine (811)



4-O-Desacetylvinblastine (769)



Vinorelbine (779)

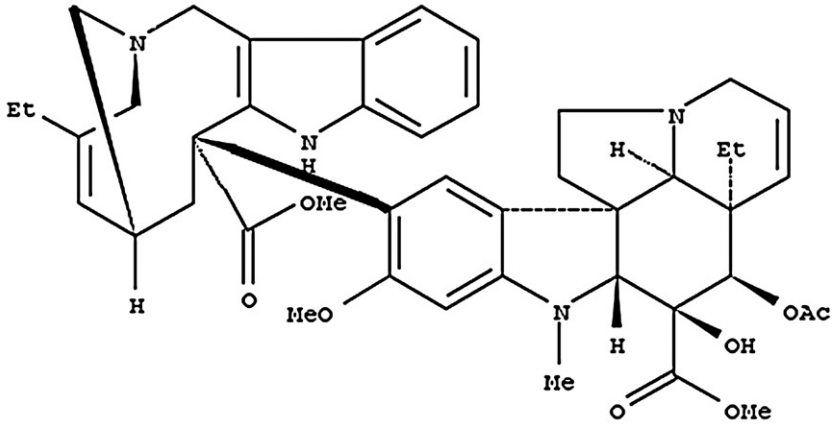


Fig. 1. Chemical structures of vinblastine, desacetylvinblastine, and vinorelbine with molecular weights in parenthesis.

vinblastine by modifying several previously published methods for the analysis of vinca alkaloids [13–15]. In LC/MS/MS, monitoring precursor drug mass and product ion fragment mass gives high specificity in the quantitation of a drug. Sensitivity of the LC/MS/MS is also higher than that of other analytical methods. The use of LC/MS/MS is becoming more prevalent in drug metabolism and *in vitro* screening of new chemical entities (NCEs) in drug development process because of its high sensitivity and specificity [16].

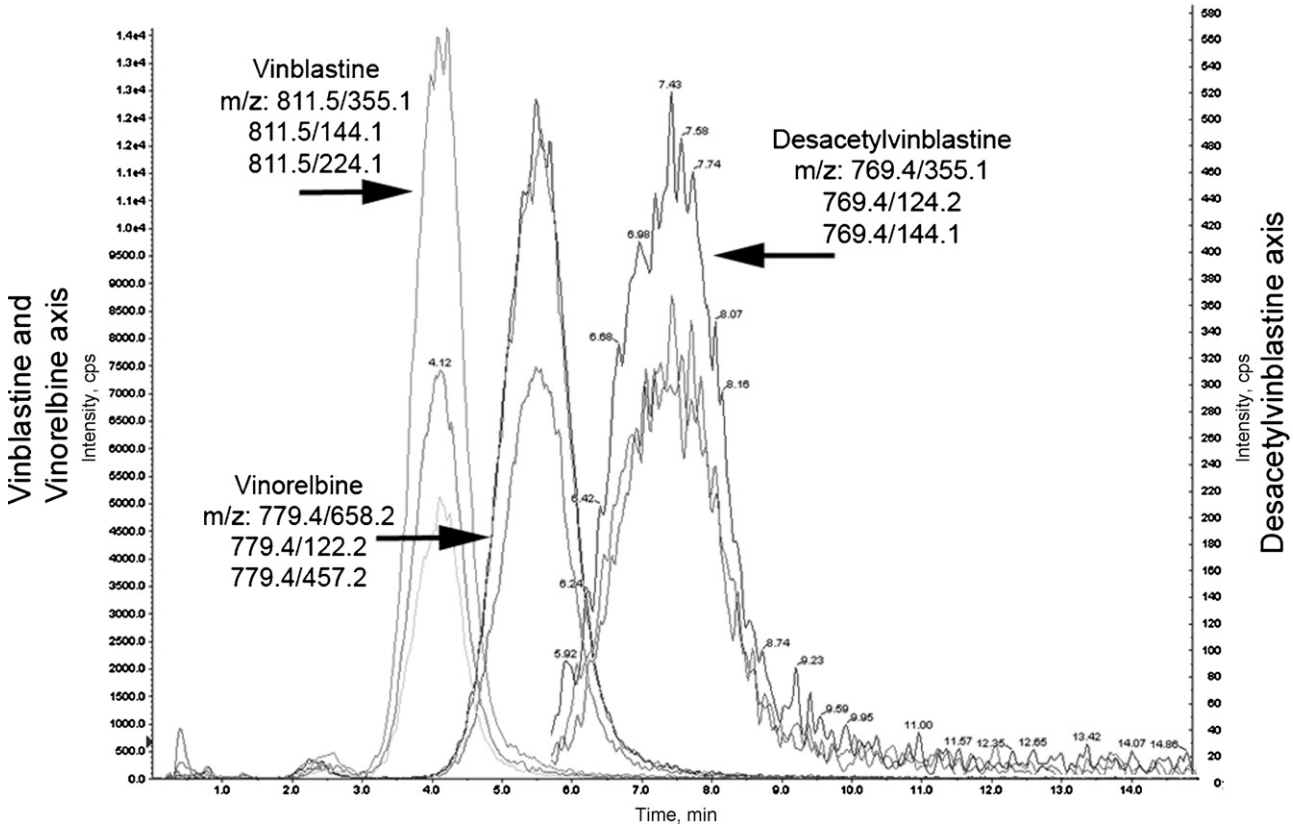


Fig. 2. Representative SRM chromatogram of canine plasma 30 min after vinblastine administration showing the specificity of vinblastine identification in plasma samples. This plasma sample was quantitated at 5.97 ng/mL of vinblastine and 0.82 ng/mL of desacetylvinblastine. Vinorelbine is the internal standard.

Several methods involving the use of mass spectrometry detection have been reported for the analysis of vinca alkaloids in biological matrices [13–15,17]. However, none of the previously published assays were reported to sensitively and specifically quantitate both vinblastine and its metabolite, 4-O-deacetylvinblastine, in plasma and urine. The assay described here provides information on a novel analytical technique and its application to the analysis of multiple biological matrices. A highly sensitive and specific liquid chromatography/atmospheric pressure chemical ionization–tandem mass spectrometry (LC–APCI–MS) analytical method was developed to simultaneously quantify vinblastine and desacetylvinblastine concentrations in canine plasma and urine samples. The developed method was successfully applied to the *in vivo* pharmacokinetic study of vinblastine in dogs.

2. Materials and methods

2.1. Chemicals and materials

Vinblastine sulfate ($C_{46}H_{58}N_4O_9{\cdot}H_2SO_4$) (Fig. 1) solution (1 mg/mL) was purchased from APP Pharmaceuticals LLC, Schaumburg, IL, USA. Vinorelbine tartrate ($C_{45}H_{54}N_4O_8{\cdot}2C_4H_6O_6$) (Fig. 1) 10 mg/mL was purchased from Teva, North Wales, PA, USA. 4-O-desacetylvinblastine ($C_{44}H_{56}N_4O_8$) (Fig. 1) was purchased from Council of Europe, European Pharmacopeia, Strasbourg, Cedex, France. HPLC – grade ammonium acetate, methanol, and acetonitrile and silanized glassware were purchased from Fisher Scientific Company, New Jersey, NJ, USA. Oasis® HLB 1 mL solid phase extraction cartridges were purchased from Waters® Corporation, Milford, MA, USA.

2.2. Liquid chromatography/mass spectrometry

A Shimadzu HPLC system (Shimadzu corporation, Kyoto, Japan) consisted of a system controller (CBM-20A), binary solvent delivery unit (LC-20AD), in-line degasser (DGU-20A5), an auto-sampler (SIL-20AC) with thermostatic injector set at 4 °C and a column oven set at 40 °C (CTO-20AC) with a Restek Allure pentafluorophenyl propyl (PFP) 5 μm 50 × 2.1 mm column (Restek, Bellefonte, PA), and guard cartridge (Restek 5 μm, Ultra Biphenyl, 10 × 2.1 mm) were used. An Applied Biosystems 4000 Q-Trap MS/MS system (Applied Biosystems, Foster City, CA) equipped with an atmospheric pressure chemical ionization source and a NitroGen N300DR nitrogen generator was used (Peak Scientific Instruments Ltd., Paisley, United Kingdom). Analyst® 1.5 software for Windows® was used to acquire and analyze the data.

2.3. Mobile phase

The mobile phase consisted of 10% 5 mM ammonium acetate buffer, adjusted to a pH of 3.5 with glacial acetic acid, and 90% HPLC grade methanol. Separation was achieved by isocratic elution at a flow rate of 0.75 mL/min.

2.4. Preparation of stock solutions

All stock and working solutions of analytes were prepared under an externally vented fume hood per chemotherapy safe handling and administration standards [18–20]. Stock solutions and working solutions for all three analytes were prepared in methanol using silanized glassware as vinca alkaloids are known to adsorb onto glassware [11,21]. All stock solutions were stable at −20 °C for up to 9 months. Depending on the necessity of

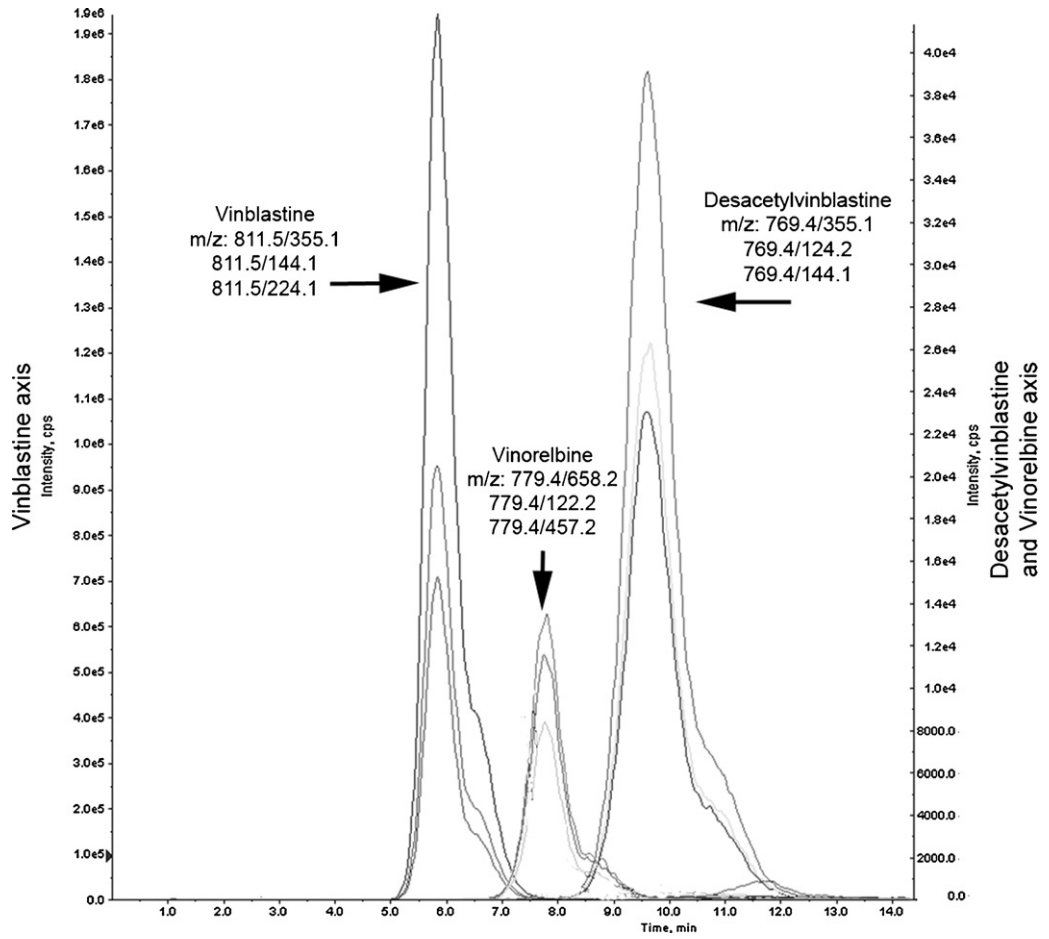


Fig. 3. Representative SRM chromatogram of canine urine 24 h after vinblastine administration showing the specificity of vinblastine identification in urine samples. This urine sample was quantitated at 606 ng/mL of vinblastine and 31.5 ng/mL of desacetylvinblastine. Vinorelbine is the internal standard.

sensitivity in each matrix, different ranges of calibrating concentrations for vinblastine and desacetylvinblastine were used. For vinblastine, a range of calibrators (6.25–5000 ng/mL for plasma samples; 500–100,000 ng/mL for urine samples) were prepared. For desacetylvinblastine, 6.25–250 ng/mL for plasma samples and 25–5000 ng/mL for urine samples were prepared. Vinorelbine was used as the internal standard; stock solution was prepared at the concentration of 100,000 ng/mL and working internal standard solution was prepared at the concentration of 500 ng/mL. All calibrators were stable for at least 3 months at 4 °C.

2.5. Sample preparation for plasma and urine

For calibrants and quality controls, 480 μL of blank plasma or urine were added to micro-centrifuge tubes. Ten micro liters of vinblastine and 10 μL of desacetylvinblastine calibrator solutions in methanol were added to achieve final concentrations of 0.125–100 ng/mL and 0.125–5 ng/mL for plasma samples; 1–2000 ng/mL and 0.5–100 ng/mL for urine samples, respectively. Incurred samples from dogs were thawed at room temperature and 500 μL of sample matrix were added to labeled micro centrifuge tubes. All samples were diluted with 500 μL water containing 10 μL of 500 ng/mL vinorelbine and vortex mixed. Oasis® HLB cartridges were preconditioned with 1 mL of methanol and 1 mL of water. Samples were loaded onto the column slowly, at a rate of approximately 1–2 drops per second. Cartridges were washed with 2 mL of water, and then dried at maximum airflow for 30 min. Samples were slowly eluted with 2 mL of methanol into labeled silanized glass tubes. The eluted samples were dried under nitrogen at 30 °C and were resuspended in 150 μL of buffer, containing 50% 5 mM ammonium acetate, 25% acetonitrile, and 25% methanol. For plasma samples, 100 μL of the redissolved sample was injected onto the system, whereas 20 μL were injected for urine samples. Silanized glassware was used wherever possible during sample preparation to avoid systemic under-estimation of analyte concentration and to increase sensitivity [6,22,23].

2.6. Calibration curve

For the quantification of vinblastine and desacetylvinblastine concentrations in experimental samples, the ratio of peak area of vinblastine or desacetylvinblastine to the peak area of the internal standard, vinorelbine, was used. For vinblastine, two calibration curves were prepared in plasma samples to obtain acceptable linearity of calibration curves, whereas only one calibration curve was used for urine samples. For desacetylvinblastine, only one calibration curve was used in both matrices. At least 6 calibrators were included for each calibration curve. Different weighting schemes were used for each analyte; $1/x$ weighting was used for high and low vinblastine calibration curves. For desacetylvinblastine, no weighting was used for plasma curves, whereas $1/x^2$ weighting was used for urine calibration curves. The best weighting model was chosen based on the accuracy of calculated concentrations and coefficient of determination (R^2) value. The limit of quantitation was defined as accuracy and coefficient of variation within 20% of nominal concentrations. Acceptability criteria for all runs included accuracy within

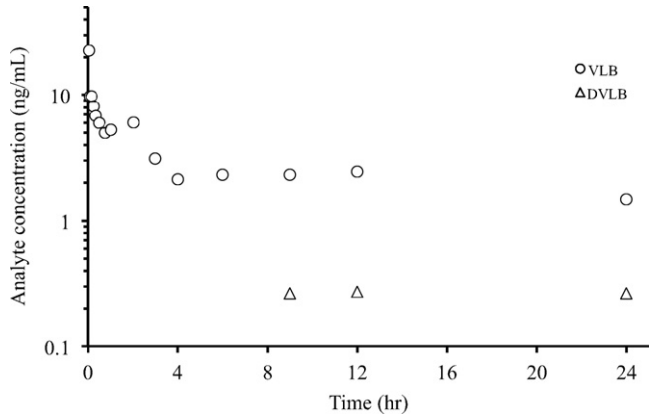


Fig. 4. A representative time course disposition of vinblastine (VLB) and desacetylvinblastine (DVLB) in a dog (54 kg body weight), that received an intravenous bolus dose of 0.075 mg/kg vinblastine sulfate.

15% in at least four concentrations in the calibration curve above the LOQ and an R^2 of 0.99 or above.

2.7. Method validation

Matrix effects, recovery efficiency, and process efficiency were determined for validation of vinblastine and desacetylvinblastine in plasma and urine. Samples were prepared in triplicate at 5 ng/mL concentration for each analyte. Matrix effect (ME) was determined as the percentage of the ratio of peak areas of analytes fortified after extraction (B) to peak areas of analytes in resuspending buffer (no extraction) (A) [24]. Recovery efficiency (RE) was calculated as the percentage of peak areas of analytes fortified before extraction (C) to peak areas of analytes fortified after extraction (B). Process efficiency (PE) was determined as the percentage of peak areas of analytes fortified before extraction (C) to peak areas of analytes in resuspending buffer (no extraction) (A).

$$\text{ME}\ \ (\%) = \frac{B}{A} \times 100$$

$$\text{RE}\ \ (\%) = \frac{C}{B} \times 100$$

$$\text{PE}\ \ (\%) = \frac{C}{A} \times 100$$

In addition to the assessment of calibration curve performance, intraday and interday accuracy and precision values were calculated in five replicate samples for each analyte in plasma and urine. The precision was calculated as the relative standard deviation (RSD), or standard deviation divided by the mean concentration, and expressed as a percentage. Accuracy within 20% of the nominal concentration at the lower limit of quantitation (LLOQ) and 15% of the nominal concentration at all higher concentrations were set as the acceptable limits. Similarly, an RSD of 20% at the LLOQ and 15% at higher concentrations were set as the acceptable parameters.

2.8. Specificity

To assess the assay's ability to measure the concentrations of each analyte distinctly in the presence of other analytes, peak areas of each analyte were compared by injecting each analyte alone and in the presence of other analytes in resuspending buffer and after extraction in sample matrices. Each analyte was injected at the LLOQ and at middle and highest concentrations used in the calibration curve. The multiple reaction monitoring (MRM) traces of each ion for each analyte and blank matrices were compared.

2.9. Sensitivity

In addition to evaluation of the lowest concentration that met accuracy and precision criteria of 20% at the LLOQ, a multiple calibration curve method, deemed the statistical method, was used to estimate the limit of detection (LOD) and LLOQ using triplicate calibrants in the range of 0.1–10 ng/mL in plasma. After linear regression of the resulting concentration *versus* response curves, the y-intercept and slope of each best fit line were used to calculate the standard deviation (SD) of the y-intercepts and the mean of the slopes of calibration curves. The LOD was calculated as (3.3 × SD of the y-intercepts)/(mean of the slopes of calibration curves). The LOQ was calculated as (10 × SD of the y-intercepts)/mean of the slopes of calibration curves [25].

2.10. Freeze–thaw stability

Freeze–thaw stability studies were performed to assess the effect of multiple freeze and thaw cycles on plasma samples. Originally, a reversed phase high performance liquid chromatography method was developed with a maximum sensitivity of 1 ng/mL. Incurred sample analysis indicated the need for a more sensitive and specific method, so the samples were frozen for later analysis, leading to the need for freeze–thaw stability tests. Urine samples were not subjected to this storage situation and therefore, freeze–thaw stability tests were not necessary. Freeze–thaw stability of plasma samples stored at −80 °C was evaluated by fortifying blank samples with vinblastine at 10 ng/mL and 50 ng/mL and desacetylvinblastine at 0.5 and 2 ng/mL, in triplicate. One batch of fortified plasma samples were frozen and thawed twice while the other batch of fortified plasma samples were thawed only once before sample processing.

2.11. Application to in vivo vinblastine pharmacokinetic study in dogs

The novel highly sensitive analytical method was successfully applied to quantify the concentrations of vinblastine and desacetylvinblastine in plasma and urine samples of four adult and two juvenile dogs (4–54 kg) that received an intravenous bolus dose of 0.075 mg/kg vinblastine sulfate. Plasma and urine samples were collected at pre, 2, 5, 10, 15, 20, 30, 45 min, 1, 2, 3, 4, 5, 6, 9, 12, and 24 h post drug administration; pre sample, 1, 3, 7, 12, 24, 32, 48, 56, and 72 h, respectively following vinblastine sulfate administration. The data was analyzed on WinNonlin® 5.2 pharmacokinetic software (Pharsight Corp., Mountain View, CA) to estimate the pharmacokinetic parameters.

3. Results and discussion

3.1. Sample pretreatment optimization

Different sample pretreatment procedures were investigated to optimize sample recovery and chromatography. Published liquid–liquid extraction procedures were investigated using different solvents such as: a mixture of hexane and ethyl acetate, chloroform and isopropanol, and ether [13]. Liquid–liquid extraction was unsuccessful due to poor recovery of analytes, base line noise, formation of by-products, appearance of degradation products, or column obstruction. Protein precipitation with acetonitrile was also attempted, but recoveries of the analytes were very poor [21]. Poor recovery of vinca alkaloids might be due to high protein binding [26]. In order to overcome different complications encountered in the aforementioned methods, solid phase extraction was examined using HLB and MCX cartridges. The solid-phase extraction (SPE) protocol using Oasis® HLB cartridges provided the

highest analyte recovery. To the authors' knowledge, the use of HLB columns in extraction of vinblastine and desacetylvinblastine has not been previously published. However, among the vinca alkaloids, the use of HLB columns has been reported for vinorelbine in several matrices [17,27]. The HLB SPE cartridges were selected for investigation in the present study because their polymeric reversed phase materials give wide versatility for use with a variety of polar and non-polar drugs and can be allowed to dry completely [28].

3.2. Chromatographic optimization

Different mobile phase compositions were explored to optimize the separation of analytes. Adequate separation and approximately Gaussian peak shape of analytes were achieved using isocratic elution with 10% 5 mM ammonium acetate (pH: 3.5) and 90% methanol at a flow rate of 0.75 mL/min. No carryover of analytes in successive injected matrix samples was observed. The absence of carryover was confirmed by injection of resuspending buffer after injection of the highest calibrant.

3.3. Instrument settings and optimization

Direct infusion of analytes was used to determine the optimal instrument parameters, such as nebulizing gas (gas1): 40; turbo ion spray (TIS, gas2): 20; temperature: 400 °C; collision energy (CE): 49 eV; collision cell exit potential (CXP): 8 V. Optimization of these parameters was important in removing or decreasing interferences in combination with chromatographic separation. Ion path for MS/MS detection of compounds was tuned by infusion of a mixture of all three analytes into the tandem mass spectrometry. For vinca alkaloids, electrospray ionization (ESI) is the most often used method of ionization. However, ESI gave low sensitivity, poor resolution, and more baseline noise compared to APCI [22]. In order to overcome these problems, atmospheric pressure chemical ionization (APCI) with positive mode ionization was used and gave satisfactory sensitivity. Few publications have described the use of APCI for the quantification of vincristine and vinblastine [29]. In APCI, ionization occurs in the gas phase, unlike ESI, where ionization occurs in the liquid phase. General advantages of APCI include high ionization efficiency, low susceptibility to chemical interferences, and also, APCI is considered to be more robust method of ionization than ESI [29,30]. Multiple reaction monitoring (MRM) was used as this is the superior method compared to single ion monitoring (SIM) [22]. A typical fragment ion is selected in MRM while a single molecular ion is selected in SIM. Methods using MRM had reported higher sensitivity than SIM mode [22].

3.4. Specificity

Specificity was verified by looking at the identification (ID) ratios of qualifier ions for each analyte. ID ratios within ±20% of mean of ID ratios were considered as an acceptable limit. The mass to charge ratios of precursor ions were 811.5, 769.4, and 779.4 for VLB, DVLB, and VRB, respectively. For quantitation of analytes, major transitions were chosen, such as 811.5/355.1 for VLB, 769.4/355.1 for DVLB, and 779.4/658.2 for VRB. For identification of analytes, qualifier transitions were 811.5/144.1 and 811.5/224.1 for VLB; 769.4/124.2 and 769.4/144.1 for DVLB; and 779.4/122.2 and 779.4/457.2 for VRB, respectively. Figs. 2 and 3 show the specificity of the developed method for the analytes of interest in canine plasma and urine samples, respectively. No interferences were found in blank plasma and blank urine samples in typical multiple reaction monitoring (MRM) traces at mass-to-charge ratio (m/z) of vinblastine and desacetylvinblastine (data not shown).

Table 1
Mean percentages of matrix effects, recovery efficiency, and process efficiency of extraction of plasma and urine samples fortified with vinblastine, desacetylvinblastine, and vinorelbine in triplicate; fortified at the concentrations of 5 ng/mL vinblastine and desacetylvinblastine, and 10 ng/mL vinorelbine. The matrix effects, recovery efficiency, and processing of efficiency of all three analytes were lower in plasma samples compared to urine samples.

	Plasma			Urine		
	VLB	DVLB	VRB	VLB	DVLB	VRB
Matrix effects	65.7	76.5	78.0	100.7	106.6	110.5
Recovery efficiency	65.3	72.0	68.7	103.6	108.5	84.6
Processing efficiency	42.9	55.1	53.6	104.4	115.7	93.5

3.5. Calibration curve

In order to obtain linear calibration curves within acceptable limits for vinblastine, two calibration curves were prepared in plasma. A wide range of vinblastine calibrants were included (0.125–100 ng/mL) in plasma to quantify the expected concentrations of vinblastine in both adult and juvenile dogs. A similar method of quantitation of vinblastine in human plasma using LC/APCI–MS was previously described [13]. However, the LOQ and range of calibration curves reported in this paper were lower and wider than the previous work. The concentrations of desacetylvinblastine in the incurred samples were within a small range and required only one calibration curve. Criteria for acceptable calibration curves included that the estimated concentrations for each calibrator were within 80% of the nominal concentration at the limit of quantitation and within 85% of the nominal concentration at higher concentrations. At least six calibrators were retained for each calibration curve, with calibrators having back calculated values more than 15% distant from nominal concentrations removed from the curves.

3.6. Method validation

Matrix effects, recovery efficiency, and processing efficiency were calculated for each analyte in both matrices [24]. Recoveries of both vinblastine and desacetylvinblastine were lower in plasma as compared to urine (Table 1). This could be attributed to binding of both analytes to plasma proteins or to ion suppression [24,26,31,32]. The intraday and interday relative standard deviation (RSD) of vinblastine and desacetylvinblastine in both matrices were within 8.6% (Tables 2 and 3). *A priori*, acceptable accuracy was considered to be within 20% of the nominal concentration at the lower limit of quantitation (LLOQ) and 15% of the nominal concentration at all higher concentrations. Similarly, an RSD of 20% at the LLOQ and 15% at higher concentrations were set as acceptable parameters. This low value indicates the repeatability of the developed method. The lowest value of the intraday and interday accuracy of both analytes in both matrices was 89% indicating good accuracy of the developed method. For both analytes, the coefficient of determination was greater than 0.99 in all validation experiments and in incurred sample analysis. Vinblastine and 4-O-deacetylvinblastine freeze–thaw stability in plasma samples processed after single thawing and double thawing were within acceptable limits (Table 4).

3.7. Sensitivity

The sensitivity of the novel method was tested by both statistical and empirical approaches. The sensitivity of the method based on the empirical approach was higher than that predicted by the statistical approach. The LLOQ of vinblastine and desacetylvinblastine were 0.125 ng/mL and 0.125 ng/mL with the empirical approach, but were 0.45 ng/mL and 0.21 ng/mL with the statistical approach,

Table 2
Intraday accuracy and precision of analytes in plasma and urine were presented as mean ± standard deviation. The intraday accuracy and precision of both analytes in both matrices were within acceptable limits.

Analyte	Intraday precision and accuracy							
	Plasma (n = 5)				Urine (n = 3)			
	Nominal conc. (ng/mL)	Observed conc. (mean ± SD) (ng/mL)	RSD (%)	Accuracy (%)	Nominal conc. (ng/mL)	Observed conc. (mean ± SD) (ng/mL)	RSD (%)	Accuracy (%)
VLB	0.5	0.51 ± 0.03	5.7	98.4	25	25.47 ± 0.9	3.5	98.1
	2	2.19 ± 0.07	3.4	90.3	250	251 ± 5.29	2.1	99.6
	10	11.04 ± 0.53	4. 9	89.6	1500	1523 ± 64.3	4.2	98.4
	50	52.6 ± 3.31	6.3	94.8				
DVLB	0.5	0.46 ± 0.02	3.6	92.7	5	4.92 ± 0.25	5.0	98.5
	2	1.85 ± 0.16	8.6	92.2	25	26.8 ± 1.44	5.4	96.1

Table 3
The values of interday accuracy and precision of analytes in plasma and urine in seven runs over a period of 9 months and 3 runs over a period of 2 months, respectively were presented as mean ± standard deviation. The values of interday accuracy and precision of both analytes in both matrices were within acceptable limits.

Analyte	Interday precision and accuracy							
	Plasma (n = 5)				Urine (n = 3)			
	Nominal conc. (ng/mL)	Observed conc. (mean ± SD) (ng/mL)	RSD (%)	Accuracy (%)	Nominal conc. (ng/mL)	Observed conc. (mean ± SD) (ng/mL)	RSD (%)	Accuracy (%)
VLB	0.5	0.4 ± 0.02	5.1	92.8	25	25.37 ± 1.12	4.4	96.3
	2	1.84 ± 0.11	5.5	89.2	250	250.8 ± 12.5	5.0	97.5
	10	9.9 ± 0.74	7.6	93.4	1500	1588.9 ± 95	6	94.1
	50	50.1 ± 4.5	8.4	95.3				
DVLB	0.5	0.45 ± 0.02	4.7	89.6	5	4.9 ± 0.27	5.6	97.2
	2	1.86 ± 0.13	7.0	92.8	25	25.4 ± 2.0	8.1	96.1

Table 4
Freeze–thaw stability of plasma samples fortified with vinblastine and 4-O-desacetylvinblastine was tested by storing the samples at −80 °C in triplicate. One batch of samples were thawed once and another batch were thawed twice, and then samples were processed together to test the freeze–thaw stability. The analytes were stable for up to two freeze–thaw cycles.

Analyte	Freeze–thaw stability testing						
	Nominal conc. (ng/mL)	Single thaw (n = 3)			Double thaw (n = 3)		
		Mean ± SD	RSD (%)	Accuracy (%)	Mean ± SD	RSD (%)	Accuracy (%)
VLB	10	8.61 ± 0.19	2.2	86.1	9.94 ± 0.19	1.9	99.4
	50	53.9 ± 1.04	1.9	92.2	46.8 ± 1.31	2.8	93.6
DVLB	0.5	0.56 ± 0.001	1.77	88.4	0.56 ± 0.004	0.6	87.8
	2	1.89 ± 0.20	10.7	94.3	1.74 ± 0.10	5.7	86.8

respectively. Although the statistical method is useful for initial predictions of LOD and LLOQ values, the empirical method is able to more concretely define the LLOQ that meets the predetermined acceptance criteria for these values [25].

3.8. Application to in vivo vinblastine pharmacokinetic study in dogs

The vinblastine concentrations determined *in vivo* in dogs ranged from 0.8 to 51.1 ng/mL and 0.45 to 884 ng/mL in plasma and urine samples, respectively. The desacetylvinblastine concentrations ranged from 0.22 to 1.03 ng/mL and 0.25 to 31.5 ng/mL in plasma and urine samples. Fig. 4 represents a typical pharmacokinetic profile of vinblastine sulfate in dogs. As seen in Fig. 4, the vinblastine assay was able to adequately describe the plasma disposition of intravenously administered vinblastine in dogs. The concentrations of vinblastine and desacetylvinblastine were above the assay's LLOQ until 24 h after vinblastine administration in plasma samples and 72 h in urine samples. The data from the *in vivo* vinblastine pharmacokinetic study was applied to on-going *in vitro*/*in vivo* studies to develop novel dosing equations in dogs to calculate the doses of anticancer drugs more accurately.

4. Conclusion

A highly sensitive, specific, and reliable analytical method to quantify the concentrations of vinblastine and its metabolite, desacetylvinblastine, in canine plasma and urine samples using liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry was developed and validated. This newly described assay is highly reproducible. The high sensitivity of the method would be particularly useful in determining the concentrations of vinblastine and its metabolite, desacetylvinblastine in small sized dogs and to study the pharmacokinetics of vinblastine and desacetylvinblastine in dogs. The analytical method developed could be useful in the toxicity adjusted dosing of vinblastine in dogs.

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