ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
16 (1997) 675–687

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Validation of a high-performance liquid chromatographic assay method for quantification of total vincristine sulfate in human plasma following administration of vincristine sulfate liposome injection

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Received 1 November 1996; received in revised form 6 March 1997

Abstract

The validation of a high performance liquid chromatographic (HPLC) assay method for quantitation of total vincristine sulfate (VINC) in human plasma is described. VINC was extracted from plasma using BondElut CBA solid phase cartridges with vinblastine as the internal standard. Chromatography was accomplished using a Waters Symmetry C8 (250 mm × 4.6 mm i.d.) analytical column, a Waters Delta-Pak ODS guard column with a mobile phase of 34.9% water–0.1% diethylamine (pH 7.0)–40% acetonitrile–25% methanol pumped isocratically at 1.0 ml min^{-1} with ultraviolet detection at 297 nm. Above the limit of quantitation of 28.6 ng ml^{-1}, the area ratio precision (R.S.D. range 3.33–11.6%) and accuracy of predicted values (R.S.D. range 8.56–23.8% with the limit of quantitation being the only value above 20%) were acceptable. The assay was linear from 28.6–2860 ng ml^{-1} VINC in plasma. Recovery of VINC from plasma and VINC from plasma spiked with vincristine sulfate liposome injection ranged from 74.9–87.1%. Stability of VINC in plasma stored at −20°C for at least 49 days and of extracted plasma samples was demonstrated. Potential interference in quantitation of VINC from commonly co-administered drugs was evaluated along with day-to-day variability. The assay procedure was found suitable for evaluation of VINC clinical pharmacokinetics in plasma following administration of vincristine sulfate liposome injection prepared using distearoylphosphatidylcholine (DSPC)/cholesterol liposomes for injection. © 1997 Elsevier Science B.V.

Keywords: Vincristine sulfate; Liposome encapsulated vincristine sulfate; Reversed-phase HPLC

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PII S0731-7085(97)00087-3

1. Introduction

Vincristine (VINC) is a dimeric catharanthus alkaloid used in the treatment of various neoplastic diseases. Differences in the clinical efficacy, toxicity and pharmacokinetics [1] of vinca alkaloids exist despite similarities in structure as shown in Fig. 1. Although VINC has been in clinical use since the 1960s, its pharmacokinetics have received limited study due to the lack of assay methods with sufficient specificity and sensitivity. Assay methods for VINC quantitation in biological fluids reported in the literature use radiolabeled VINC [2], immunoassay [3–7] or high performance liquid chromatography (HPLC) [8–12]. Both the use of radiolabeled VINC and immunoassay procedures offer sufficient sensitivity but specificity is a serious concern [2–7]. For radiolabeled VINC, specificity is a concern as the label remains associated with the metabolites [2,13]. Specificity can be attained by HPLC techniques to isolate VINC from potential degradation products and endogenous components of biological fluids [8,14,15]. However, electrochemical detection has been required to provide the sensitivity needed for pharmacokinetic evaluation of VINC and other vinca alkaloids in vivo [14,15]. This approach is complicated by the fact that HPLC methods using electrochemical detection are both difficult to optimize and problematic to maintain due to their sensitivity towards the sample and chromatographic conditions [16–18].

A selective assay for VINC in human plasma is required to support the pharmacokinetic evaluation of the liposome encapsulated VINC formulation vincristine sulfate liposome injection (VSLI) for Phase I clinical testing. This Phase I clinical study has been designed to define the maximum tolerated dose of VSLI and the spectrum of toxicity as well as the pharmacokinetic behaviour of VINC when administered intravenously (i.v.) in an encapsulated form. The liposomes being utilized for this clinical study are composed of distearoylphosphatidylcholine (DSPC) and cholesterol at a molar ratio of 55:45 and are processed to obtain a homogeneous size distribution with a mean diameter of approximately 120 nm [19].

Clinical evaluation of VSLI was initiated based on its improved therapeutic activity compared to conventional VINC in several animal models [19–22]. Previous preclinical studies with this and other liposomal formulations suggest that potential liposome related improvements in the toxicity or efficacy properties of VINC will arise from altered drug pharmacokinetic and tissue distribution properties [20,23]. Animal studies have demonstrated that small (less than 200 nm) liposomes capable of retaining entrapped VINC after i.v. injection reduced the accumulation and subsequent damage to healthy tissues, resulting in a modest reduction in drug toxicity [20,23]. Of more significance is the dramatic increase in the antitumour potency of VINC when encapsulated in liposomes [19–22,24]. Such increases in potency correlate with plasma VINC concentrations which are 100- to 1000-fold higher than those observed after administration of the free drug over 48 h post administration [21,24]. These elevated plasma concentrations are also associated with increased and extended exposure of tumours to VINC in vivo [21,24].

The pharmacodynamic correlations described above for preclinical studies were possible only through the use of high specific activity prepara-

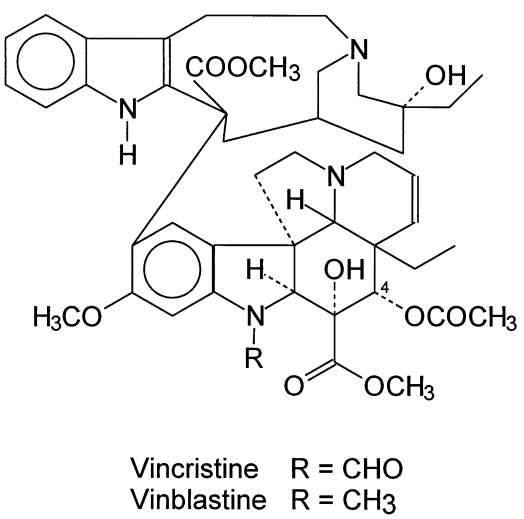

Fig. 1. Structures of the vinca alkaloids vincristine and vinblastine.

tions of tritiated VINC and combining the use of radiolabeled tracer with HPLC to assess specificity of radioactivity present in plasma for VINC [19]. Such approaches to evaluate the pharmacokinetic properties of liposome encapsulated VINC in a clinical setting would be extremely difficult if not impossible to perform from a technical perspective and presents serious ethical concerns. Based on the preclinical observations, the use of a liposomal formulation will likely provide extended exposure to VINC in patients receiving VSLI. It is highly improbable that the selectivity of an antibody or radiolabel-based assay procedure for VINC will be sufficient at extended plasma sampling times (24 h and beyond) to allow reliable determination of the pharmacokinetic parameters of intact VINC. Consequently, we have developed and validated an HPLC assay procedure using ultraviolet detection for quantitation of VINC in human plasma that provides the desired selectively with sufficient sensitivity to quantitate VINC following administration of VSLI.

This report describes an HPLC assay method for total VINC in human plasma using ultraviolet detection. This assay method was validated by characterization of the following parameters: precision, accuracy, linearity, limit of quantitation, extraction recovery of VINC from plasma, recovery of VINC from VSLI in plasma, stability of stored extracted plasma samples stored at −20°C, stability of VINC in plasma, specificity and ruggedness (as estimated by day-to-day variability).

2. Materials and methods

2.1. Materials

Reagent grade diethylamine, potassium phosphate, monobasic (anhydrous), sodium phosphate dibasic anhydrous, *o*-phosphoric acid, and HPLC grade phosphoric acid, acetonitrile and methanol were obtained from Fisher Scientific (Vancouver, BC). HPLC grade water was prepared fresh daily with the Milli-Q system (Millipore, Bedford, MA). Durapore 0.45 μm HV HP filters used during mobile phase preparation were obtained from Millipore. Ammonium hydroxide (97–99%) was purchased from Aldrich (Milwaukee, WI). Potassium chloride was obtained from Mallinckrodt Canada (Pointe-Claire, Quebec). Undenatured ethanol (100%) was purchased from Stanchem (Vancouver, BC). Vinblastine sulfate USP was obtained from A + C American Chemicals (Toronto, Ont) for use as the internal standard. The drugs indicated in Table 1 were used to evaluate assay specificity.

2.2. Chromatographic conditions

The HPLC system considered of a Waters 710B autosampler, a 510 solvent delivery system, a 481 detector, the Millennium Version 2.10 software used on a NEC Image 466es computer, a Symmetry C8 (250 mm × 4.6 mm i.d.) analytical column with a Delta-Pak C18 guard column (Waters Associates, Milford, MA). The mobile phase consists of 34.9% HPLC grade water–0.1% diethylamine (to pH 7.0 with *o*-phosphoric acid)–40% acetonitrile–25% methanol and was used at a flow rate of 1.0 ml min^{-1}. The detection wavelength was 297 nm and the injection volume was 70 μl. Between each injection of plasma extract, a 1 ml volume of methanol was injected to remove endogenous components that were retained on the column.

2.3. Preparation of VSLI

VSLI, 0.16 mg ml^{-1}, is a three part formulation consisting of DSPC/cholesterol liposomes for injection, 100 mg ml^{-1}, sodium phosphate for injection, 14.2 mg ml^{-1} and Oncovin (vincristine sulfate for injection from Eli Lilly Canada). Both the DSPC/cholesterol liposomes for injection, 100 mg ml^{-1}, and sodium phosphate for injection, 14.2 mg ml^{-1} used in this study were prepared according to Good Manufacturing Practices by the Medical Oncology Investigational Drug Section (IDS) at the British Columbia Cancer Agency (Vancouver, BC). Oncovin was obtained from Eli Lilly and used without further modification. The combination of these three agents to form VSLI was used to characterize the recovery of VINC

Table 1
Drugs evaluated for potential interference with quantitative analysis of VINC

Drug name (supplier)	Formulation	Concentration evaluated
Hydroxymorphone (Dilaudid) (Knoll Pharmaceuticals Canada, Markham, Ont.)	Injection (2 mg ml^{-1})	2.5 mg l^{-1}
Tylenol #3 (McNeil Pharmaceutical (Canada) Stouffville, Ont.)	Tablet (300 mg acetaminophen, 30 mg tablet codeine and 15 mg caffeine)	Equivalent of 1 tablet 8 l^{-1}
Ciprofloxacin HCl monohydrate (Miles Canada, Etobicoke, Ont.)	Tablet (500 mg)	500 mg 8 l^{-1}
Hydrochlorothiazide/amiloride (Apotex, Westeron, Ont.)	Tablet (50 mg 5 mg^{-1})	Equivalent of 1 tablet 8 l^{-1}
Ranitidine (Novopharm, Scarborough, Ont.)	Tablet (150 mg)	150 mg 8 l^{-1}
Docusate sodium (Technilab, Saint-Laurent, Que.)	Liquid (20 mg 5 ml^{-1})	180 mg 8 l^{-1}
Glysennid (sennosides) (Pharmascience, Montreal, Que.)	Tablet (8.6 mg)	8.6 mg 8 l^{-1}
Prednisone (Novopharm)	Tablet (5 mg)	5 mg 8 l^{-1}
Heparin (Organon Teknika, Scarborough Ont.)	Injection (10000 I.U./ml)	20000 I.U. 8 l^{-1}
Lorazepam (ativan) (Wyeth-Ayerst Canada, North York, Ont.)	Injection (4 mg 1 ml^{-1})	2 mg 8 l^{-1}
Salbutamol (ventolin) (Glaxo Canada, Toronto, Ont.)	Inhaler solution (1 mg ml^{-1})	400 μg 8 l^{-1}
Ipratropium bromide (atrovent) (Boehringer Ingelheim (Canada), Burlington, Ont.)	Inhaler (20 μg per actuation)	160 μg 8 l^{-1}
Beclomethasone dipropionate (beclovent) (Glaxo)	Inhaler (50 μg per actuation)	1 mg 8 l^{-1}
Anileridine (leritine) (Merck Frosst Canada, Pointe-Claire-Dorval, Que.)	Injection (25 mg ml^{-1})	200 mg 8 l^{-1}
Oxazepam (novopharm)	Tablet (15 mg)	100 mg 8 l^{-1}
Loperamide HCl (imodium) (Janssen Pharmaceutica, Mississuaga, Ont.)	Liquid (0.2 mg ml^{-1})	16 mg 8 l^{-1}
Dexamethasone (Sabex, Bouchervill, Que.)	Injection (4 mg ml^{-1})	20 mg 8 l^{-1}
Indomethacin (indocid) (Merck Sharp and Dohme Canada, Kirkland, Que.)	Capsules	150 mg 8 l^{-1}
Desyrel (trazodone) (Bristol-Myers Squibb, Pharmaceutical Group, Montreal, Que.)	Tablet (50 mg)	200 mg 8 l^{-1}
Terbutaline (Astra Pharma, Mississauga, Ont.)	Tablet (5 mg)	15 mg 8 l^{-1}
Sotacor (sotalol) (Bristol-Myers Squibb)	Tablet (160 mg)	400 mg 8 l^{-1}
Enalapril (Merck Frosst)	Tablet (5 mg)	40 mg 8 l^{-1}
Morphine sulfate (Rhone-Poulenc Rorer Canada, Montreal, Que.)	Capsule (10 mg)	100 mg 8 l^{-1}
Cimetidine (tagamet) (SmithKline Beecham Pharma, Oakville Oak.)	Injection (1250 mg ml^{-1})	2000 mg 8 l^{-1}
Naproxen (naprosyn) (Syntex, Mississauga, Ont.)	Suspension (25 mg ml^{-1})	500 mg 8 l^{-1}
Cyclobenzaprine (Merck Frosst)	Tablet (10 mg)	40 mg 8 l^{-1}
Ibuprofen (apotex)	Tablet (300 mg)	1200 mg 8 l^{-1}
Verapamil (Novopharm)	Tablet (80 mg)	800 mg 8 l^{-1}
Dimenhydrinate (Sabex)	Injection (50 mg ml^{-1})	100 mg 8 l^{-1}
Erythromycin (Novopharm)	Injection (1 g vial)	1000 mg 8 l^{-1}
Imipenem (primaxin) (Merck Sharp and Dohme)	Injection (500 mg vial)	500 mg 8 l^{-1}
Furosemide (Sabex)	Injection (10 mg ml^{-1})	200 mg 8 l^{-1}
Prochlorperazine (Sabex)	Injection (5 mg ml^{-1})	30 mg 8 l^{-1}
Metoclopramide (Wyeth-Ayerst)	Injection (5 mg ml^{-1})	100 mg 8 l^{-1}
ASA (Bayer, Sterling Winthrop, Markham, Ont.)	Tablet (325 mg)	1000 mg 8 l^{-1}
Estazolam (Abbott Laboratories, Montreal, Que.)	Tablet (2 mg)	1 mg 8 l^{-1}
Cortisone (Merck Sharp and Dohme)	Injection (50 mg ml^{-1})	100 mg 8 l^{-1}

from plasma samples containing VSLI. The encapsulation procedure involved addition of 1 ml vincristine sulfate injection, 1 mg ml^{-1} and 0.2 ml DSPC/cholesterol liposomes for injection, 100 mg ml^{-1}, to a sterile vial and mixing (by inverting the vial 5 times). Sodium phosphate for injection, 14.2 mg ml^{-1} buffer solution (5 ml) was then added and the sample was mixed. The mixture was heated for 5 min at 63°C (60–65°) and mixed. The mixture was heated for an additional 5 min and mixed again. For the analytical work described here, the volumes of each solution indicated above were used. This encapsulation procedure was used by the pharmacists for preparation of the VSLI for administration to patients.

2.4. Preparation of reagents

The reagents required for extraction are phosphate buffered saline (PBS) and ammonium hydroxide in methanol (0.1%, v/v). The PBS was prepared fresh every month by dissolving 32.0 g sodium chloride (ACS reagent grade, Fisher Scientific), 0.8 g potassium chloride, 4.6 g sodium phosphate, dibasic, and 0.8 g potassium phosphate, monobasic in 4 l of distilled water. The resulting solution was adjusted to pH 7.2 using 10 N potassium hydroxide. Ammonium hydroxide in methanol was prepared by dissolving ammonium hydroxide (0.1 ml) in approximately 50 ml of methanol and making up to a final volume of 100 ml with methanol.

2.5. Preparation of standards

USP reference standard vincristine sulfate (USPRSVINC) was used for preparation of standard curve samples and during validation of the assay. The water content of USPRSVINC was established as described in the USP XXII using thermogravimetric analysis followed by determination of the exact dry weight of aliquots in 10 ml volumetric flasks containing approximately 10 mg. This dry weight was subsequently used to determine the exact VINC content for the solutions prepared as described below.

The standard curve samples of VINC in drug-free plasma (collected using citrate–phosphate–dextrose as anticoagulant) (Canadian Red Cross, Vancouver, BC) required three working standards of USPRSVINC which were prepared from an initial stock solution (approximately 10 mg of USPRSVINC made up to a final volume of 10 ml with HPLC grade water). This stock solution (approximately 10 mg ml^{-1}) was serially diluted with water to approximately 10 000, 1000 and 100 ng ml^{-1} to generate working standards A, B, and C, respectively.

Vinblastine sulfate USP (VINB) was used as the internal standard (IS) for quantitation of VINC and was prepared by dissolving approximately 10 mg VINB in 5 ml HPLC grade water and further diluting this solution 100-fold with HPLC grade water to a final concentration of approximately 0.02 mg ml^{-1}.

2.6. Preparation of plasma standard curve samples

Standard curves consisting of plasma samples containing USPRSVINC were prepared by addition of working standards A, B, and C to blank plasma (0.5 ml) in 16 mm × 100 mm tubes to final concentrations of approximately 3, 5, 10, 30, 50, 100, 300, 500, 1000 and 3000 ng ml^{-1}. Accurate concentration values for each standard were calculated based on the dry weight of the USPRSVINC used.

An aliquot (20 μl, approximately 400 ng) of the final dilution of IS was also added to all plasma standards (with the exception of blank plasma samples containing no VINC) to allow quantitation using peak area ratio values (VINC area/IS area). Phosphoric acid (50 μl 0.5 M aqueous solution) and 2 ml ice cold 95% ethanol were added to each sample. After vortexing for 15 s, samples were centrifuged for 5 min at $600 \times g$ and the supernatant transferred to a clean 16 mm × 100 mm test tube. PBS was added to each sample followed by vortex mixing for 5 s and transfer to the solid phase extraction cartridges prepared as descried below.

2.7. Plasma extraction procedure

Solid phase extraction cartridges (CBA BondElut, Varian, Harbour City, CA) were used for preparation of plasma samples prior to HPCL analysis. The cartridges were first conditioned with 2 aliquots of HPLC grade methanol (1 ml) followed by 2 aliquots of PBS (1 ml). The eluent was discarded and plasma samples (0.5 ml) were applied to separate cartridges. The samples were pulled through the cartridge with a slight vacuum and the cartridges were washed with 5 1 ml aliquots of PBS (pH 7.2). The cartridges were dried under vacuum for 3–5 min and the sample eluted with 1 ml 0.1% ammonium hydroxide in HPLC grade methanol. Samples were dried under a stream of nitrogen gas at approximately 40°C and stored at −20°C until analysis. All plasma extracts were reconstituted with 100 μl HPLC grade water, vortex mixed for 15 s and transferred to autosampler vial inserts just prior to HPLC analysis.

2.8. Retention time and area ratio precision

Retention time precision was evaluated by analysis of plasma samples containing known amounts of both VINC and IS (four plasma standard curves) on 1 day.

The precision of area ratio values was determined on 4 separate days by evaluation of 4 standard curves on 1 day and 4 selected concentrations in quadruplicate on 3 additional days.

2.9. Accuracy

The accuracy of the assay method for quantitation of VINC in plasma was evaluated using the data from analysis of four standard curves in plasma described above. Data with acceptable precision of area ratio values was used for determination of accuracy (i.e. data with an R.S.D. of 25% or less; concentrations from 28.6–2860 ng ml^{-1}). Least squares linear regression analysis was used to evaluate each curve. Predicted concentration values were calculated with the remaining three curves. In this manner, a total of 12 predicted values were possible at each concentration.

2.10. Linearity

The linearity of the assay procedure was determined by calculation of a regression line using the method of least squares analysis and by investigating the accuracy of the assay method as described above.

2.11. Limit of quantitation

The limit of quantitation for this assay procedure was investigated by evaluation of the accuracy and precision of analysis of plasma samples containing small amounts of VINC in plasma, namely 2.86, 5.72, 11.44, 28.6 and 57.2 ng ml^{-1}.

2.12. Extraction recovery of VINC from plasma

To evaluate the recovery of VINC from plasma samples using the extraction procedure described above, plasma samples containing oncovin (at 96 and 960 ng ml^{-1}) were prepared in triplicate as described below. Oncovin (1 mg ml^{-1} VINC) was diluted 100-fold with drug free plasma (concentration of final solution 10 ng μl^{-1}). A second concentration was prepared by further dilution of this 10 ng μl^{-1} solution 10-fold with plasma (concentration of final solution 1.0 ng μl^{-1}). Samples at 96 and 960 ng ml^{-1} were prepared in triplicate by addition of 48 μl of the 1.0 and 10 ng μl^{-1} solutions to plasma (0.5 ml), respectively. Following extraction, these samples were reconstituted with 20 μl of IS (approximately 400 ng) and 80 μl of HPLC grade water (final volume 100 μl). The IS was added just prior to HPLC analysis (i.e. the IS was used as an external standard) to allow direct comparison with non-extracted samples. Aqueous oncovin solutions at 10 and 1.0 ng μl^{-1} were also prepared by dilution of oncovin (1 mg ml^{-1}) 100-fold with HPLC grade water (10 ng μl^{-1}) and further dilution 10-fold with HPLC grade water (1.0 ng μL^{-1}). Non-extracted samples containing 960 and 96 ng ml^{-1} were prepared in triplicate by mixing 48 μl of each aqueous oncovin solution (10 and 1.0 ng μl^{-1}), 20 μl IS solution and 32 μl HPLC grade water.

2.13. Recovery of VINC from VSLI in plasma

To evaluate the recovery of VINC from plasma samples containing VSLI using the extraction procedure described above, plasma samples containing VSLI (at 96 and 960 ng ml^{-1}) were prepared in triplicate using the following procedure. As described above, the IS was added just prior to HPLC analysis (i.e. the IS was used as an external standard) to allow direct comparison with non-extracted samples. VSLI (0.16 mg ml^{-1} VINC) was prepared using DSPC/cholesterol liposomes for injection (IDS Lot No. IC009). The VSLI was diluted ten-fold with drug free plasma (final concentration 16 ng μl^{-1}). Further dilution of this solution (16 ng μl^{-1}) ten-fold with drug free plasma provided a final concentration of 1.6 ng μl^{-1}. Samples containing VSLI (96 and 960 ng ml^{-1} VINC) were prepared in triplicate by the addition of 30 μl of the 1.6 and 16 ng μl^{-1} solutions of 0.5 ml plasma, respectively. Following extraction, these samples were reconstituted with 20 μl IS and 80 μl HPLC grade water (final volume 100 μl). The plasma samples were prepared and assayed along with the non-extracted samples described above.

2.14. Stability of stored extracted plasma samples at ±20°C

Plasma standard curve samples containing VINC and IS were extracted and the extract stored at −20°C, from 1–6 days.

2.15. Stability of VINC in plasma

The stability of VINC in plasma was evaluated following storage at −20°C, 4°C and room temperature (24°C). Two sets of plasma samples containing VINC at approximately 100 and 1000 ng ml^{-1} were prepared in quadruplicate. One set was assayed immediately and the other was stored at −20°C for 49 days prior to analysis. To further evaluate the stability of VINC in plasma, samples containing approximately 300 ng ml^{-1} were prepared in quadruplicate for storage at −20°, 4°C and room temperature (24°C). Following storage of VINC in plasma under these conditions for 14 days, all samples were assayed as described above.

2.16. Specificity

The specificity of the assay procedure was evaluated by HPLC analysis of blank plasma, co-administered drugs and other vinca alkaloids. Drug free plasma samples to which neither VINC nor IS were added were extracted and evaluated with each of four standard curves in 1 day as described for precision above. Fig. 2 shows a representative chromatogram for blank plasma.

The potentially co-administered drugs that were analyzed using the chromatographic assay method are described in Table 1. Aqueous samples of all drugs corresponding to the concentrations indicated were prepared. Solid dosage forms were weighed, crushed and a portion of the powder was dissolved in water to provide the desired concentration. Liquid dosage forms were diluted with water to the desired concentration. All samples (the supernatant of suspensions) were injected directly into the HPLC and evaluated as described for plasma samples. For leratine and verapamil, additional samples were evaluated containing these compounds plus VINC and IS. Plasma samples containing approximately 0.4 mg ml^{-1} of verapamil (with and without IS) were also analyzed using the assay procedure described above.

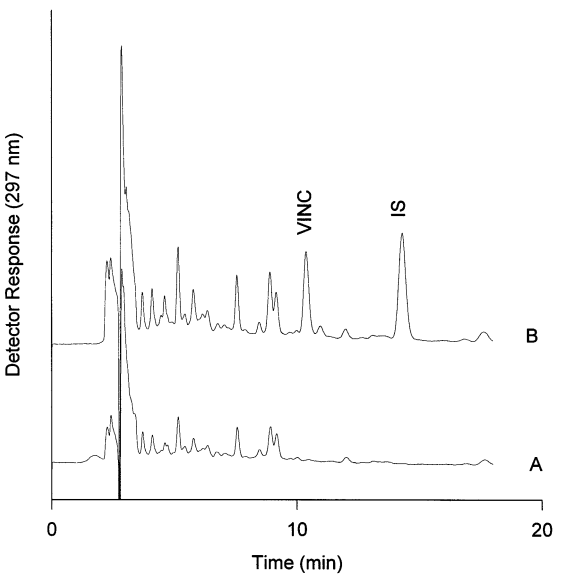

Fig. 2. Representative chromatograms of (A) blank plasma and (B) plasma containing VINC (327 ng ml^{-1}) and IS.

2.17. Ruggedness (as estimated by day-to-day variability)

The ruggedness of the assay procedure was determined by estimation of the day-to-day variability observed in the peak area ratio data obtained from evaluation of four standard concentrations of VINC in plasma in quadruplicate on 3 separate days.

3. Results and discussion

3.1. HPLC assay development

Improvements to previously reported HPLC assay methods for VINC [11,15] were based on enhancing the sensitivity and increasing the standard curve range so dilution of plasma samples would not be required. The plasma extraction procedures reported by Vendrig et al. [14,15] were modified to enable recovery of total VINC from VSLI in plasma by optimization of the stationary phase, wash procedures and eluting solvent.

Because of inclusion of liposomal lipid in the plasma to be evaluated, additional interference in the chromatographic resolution and/or solid phase extraction of VINC was a consideration in assay development. The precision of the assay and chromatographic behaviour of VINC (as described below) were similar to reports of these parameters in the literature for conventional VINC [11,14] suggesting that the anticipated interference was effectively prevented by the extraction procedure and reproducible sample handling. Compared to the other HPLC assay procedures for VINC utilizing ultraviolet detection that report limit of detection values [9,11] the assay method reported here has the ability to detect lower concentrations of VINC in plasma (2.86 ng ml^{-1} compared to 6–9 ng ml^{-1}). This is likely due to differences in detection wavelength, analytical columns and the method used for preparing biological samples prior to chromatographic analysis.

Table 2
Peak area ratio precision statistics for standard curve data

Actual concentration (ng ml^{-1})	Mean (n)	S.D.	R.S.D. (%)
2.86	0.0206 (3)	0.0112	54.4
5.72	0.0318 (3)	0.0227	71.4
11.4	0.0332 (4)	0.0139	41.9
28.6	0.0713 (2)	0.00240	3.33
57.2	0.131 (4)	0.0124	9.47
114	0.241 (3)	0.0112	4.65
286	0.5240 (4)	0.0314	6.04
572	1.08 (4)	0.0754	6.98
1140	2.15 (4)	0.128	5.95
2860	5.35 (4)	0.335	6.26

3.2. Precision

The precision of the retention times observed for VINC ($n = 35$) and IS ($n = 37$), expressed as R.S.D., were 1.4 and 5.5%, respectively. Retention time precision was found to be acceptable [25] and will allow identification of the peaks corresponding to VINC and IS. The representative chromatograms of blank plasma and plasma containing VINC plus IS shown in Fig. 2 indicate that there are no endogenous interfering components co-eluting with either VINC or the IS.

Peak area ratio precision R.S.D. values described in Table 2 ranged from 71.4–3.33% for the complete range of concentrations evaluated in plasma (2.86–2860 ng ml^{-1}). The R.S.D. values obtained from the data collected in quadruplicate on 3 separate days at concentrations of 32.7, 65.3, 327 and 1310 ng ml^{-1} in plasma ranged from 3.86–11.6% (Table 3). The acceptable precision for peak area ratio values is 15% R.S.D., except for the limit of quantitation where values up to 20–25% are acceptable [25]. Therefore, concentration values from 28.6–2860 ng ml^{-1} VINC in plasma were found to have acceptable precision of peak area ratio values. The precision of the assay for samples of 2.86, 5.72 and 11.4 ng ml^{-1} was not acceptable. The current procedure is not as precise as previous reports in the literature using HPLC-UV for quantitation of VINC in serum or plasma [11,14], however, this assay quantitates total VINC over a concentration range that is from 3- to 19-fold greater.

Table 3
Peak area ratio precision statistics for analysis on 3 separate days

Actual concentration (ng ml^{-1})	Day	Mean (n)	S.D.	R.S.D. (%)
1310	1	2.21 (4)	0.111	5.02
1310	2	2.41 (3)	0.151	6.27
1310	3	2.86 (4)	0.114	3.99
327	1	0.586 (4)	0.0681	11.6
327	2	0.571 (3)	0.0420	7.36
327	3	0.657 (4)	0.0559	8.51
65.3	1	0.0942 (4)	0.00354	3.86
65.3	2	0.0155 (3)	0.00731	4.72
65.3	3	0.103 (4)	0.0105	10.2
32.7	1	0.0495 (4)	0.00427	8.63
32.7	2	0.0147 (4)	0.00911	6.20
32.7	3	0.0621 (4)	0.00548	8.82

3.3. Accuracy

Evaluation of the accuracy of the assay procedure for determination of total VINC in plasma was based on extracted plasma samples which demonstrated acceptable precision (concentrations from 28.6–2860 ng ml^{-1}). The accuracy of the assay is the closeness of the test results to the actual or true value. Accuracy was determined by evaluating plasma samples containing known amounts of VINC and using calibration curves to predict concentration values. The acceptable accuracy for predicted concentration values for pharmacokinetic assay procedures is 15% R.S.D. [25] except for the limit of quantitation where values of 20–25% are also acceptable. The R.S.D. observed for concentrations from 28.6 to 2860 ranged from 8.56 to 23.8 with 28.6 ng ml^{-1} being the only concentration value above 20% (Table 4). The accuracy of the assay procedure was found acceptable over plasma concentrations from 28.6 to 2860 ng ml^{-1}. Other HPLC-UV methods for quantitation of VINC in plasma or serum have not reported the accuracy of the assay procedure for predicting VINC concentration [11,14].

3.4. Linearity

The linearity of the assay procedure was evaluated by least squares regression analysis and by investigating the accuracy as described above. The accuracy of the method demonstrates that the linear equation used to generate predicted values provides results that are proportional to the actual concentration values. The correlation coefficients provided in Table 5 are greater than this value (range from 0.9998 to 0.9999) demonstrating that the assay method is linear and acceptable for quantitative analysis [25]. Both regression analysis and the accuracy of predicted vales show that the assay method is linear over the concentration range evaluated. Reports in the literature describing HPLC assay procedures for VINC [11,14,15] also described linear calibration but evaluated much smaller concentration ranges than described here.

3.5. Limit of quantitation

The limit of quantitation is the lowest concentration that can be determined with acceptable precision and accuracy using the assay procedure. As described above for VINC in plasma, the lowest concentration with acceptable precision and accuracy is 28.6 ng ml^{-1} in plasma with R.S.D. of 3.33 and 23.8% for peak area ratio precision and accuracy of predicted values, respectively. The limit of quantitation of the assay procedure for determination of VINC in plasma is 28.6 ng ml^{-1}. Lower limits of quantitation for VINC in plasma have been described for assay procedures using more sensitive detection methods [11,14,15] but were not specifically evaluated for other HPLC-UV methods [9].

Table 4
Summary of predicted concentration value statistics

Actual concentration (ng ml^{-1})	Mean (n)	% Bias	S.D.	R.S.D. (%)
28.6	24.6 (4)	−16.3	5.85	23.8
57.2	54.0 (12)	−5.93	10.5	19.4
114	112 (9)	−1.79	13.2	11.8
286	263 (12)	−1.79	13.2	11.8
572	566 (12)	−1.06	58.1	10.3
1140	1140 (12)	0	106	9.30
2860	2860 (12)	0	272	9.51

3.6. Recovery

The recovery of VINC from plasma shown in Table 6 was determined by comparison of the peak area ratio values for plasma samples containing 96 and 960 ng ml^{-1} VINC (oncovin) with IS added just prior to HPLC analysis (used as an external standard) and non-extracted samples. Recovery of greater than 84% was observed (87.1 and 84.1% for the 96 and 960 ng ml^{-1} samples, respectively).

The recovery of total VINC from plasma was determined by comparison of the peak area ratio values for plasma samples containing 96 and 960 ng ml^{-1} VSLI with IS added just prior to HPLC analysis (used as an external standard) and non-extracted samples. Recovery of 88.8 and 74.9% was observed for the 96 and 960 ng ml^{-1} samples, respectively. The mean peak area ratio values observed for plasma containing oncovin and VSLI are within 1 S.D. of each other indicating that recovery of VINC is the same from both samples types. Thus, unencapsulated VINC is suitable for use in calibration standards. The recovery of VINC observed here is similar to that described in the literature for other solid phase extraction [14,15], on-line column-extraction [11], and liquid-liquid extraction procedures [9].

Table 5
Least squares regression analysis of standard curve data

	Correlation coefficient	Y-intercept	Slope
Curve a	0.9999	0.0113	0.00180
Curve b	0.9999	−0.0411	0.00206
Curve c	0.9999	0.0399	0.00180
Curve d	0.9999	0.0283	0.00182

3.7. Stability

Evaluation of the effect of short term storage of extracted plasma samples on the standard curve characteristics and chromatographic behaviour of VINC and IS was also performed. Regression analysis of the standard curve data gave correlation coefficients and values for the slope and Y-intercept within the same order of magnitude following storage of extracts at −20°C from 1–6 days. The chromatographic behaviour was also unaffected by storage of extracted plasma samples.

The evaluation of VINC stability in plasma included analysis of the stability following storage at −20°C for 49 days as well as an accelerated

Table 6
Recovery of VINC from plasma containing oncovin and vincristine sulfate liposome injection

Unit (ng ml^{-1})	Recovery (%) based on mean peak area
	Ratio values
VINC (oncovin in plasma)	
96	87.1
960	84.1
VINC (from VSLI in plasma)	
96	88.8
960	74.9

Table 7
Comparison of peak area ratio values for VINC in plasma under various storage conditions

Actual concentration (ng ml^{-1})	Storage	*T*-statistic	*P*-value (two tailed)
115	−20°C for 49 days	0.338	0.757
1150	−20°C for 49 days	0.556	0.6165
327	−20°C for 14 days	0.192	0.860
327	−4°C for 49 days	2.28	0.107
327	−24°C for 14 days	4.37	0.022

stability study at three different temperatures (−20°C, 4°C and 24°C) over 14 days. The comparison of peak area ratio values of stored samples with freshly prepared samples demonstrated that there was no statistical difference for all samples stored at −20°C, and for the 327 ng ml^{-1} samples stored at 4°C (see Table 7). A statistically significant difference was observed for samples stored at 24°C for 14 days. Although this stability data is compounded by the day-to-day variability of the assay procedure, there was no significant difference for all plasma samples stored at or below 4°C for up to 49 days. As well, the mean peak area ratio values for samples stored at room temperature for 14 days (0.455 ± 0.0621) were within 1 S.D. of the mean for samples stored at 4°C (0.498 ± 0.0261) suggesting that the difference in peak area ratio values is partially due to assay variability.

3.8. Specificity

The specificity of the assay procedure is a measure of the degree of interference in the quantitation of VINC in plasma from various components in plasma. Endogenous components in plasma, co-administered drugs and other vinca alkaloids were investigated here for potential interference with the HPLC assay method.

Drug free plasma samples to which neither VINC nor IS were added consistently gave no response (peaks) at the retention times for VINC and IS as indicated by the representative blank chromatogram in Fig. 2. The co-administered drugs that would potentially be present in patient plasma were analyzed using the chromatographic assay method. Aqueous samples of all drugs corresponding to the concentrations shown in Table 1 were prepared. Evaluation of all samples demonstrated that the following drugs did not contain components that elute near either VINC or IS: docusate sodium hydrochlorothiazide/amiloride, ciprofloxacin, tylenol # 3, ipratropium bromide, salbutamol, lorazepam, heparin, dexamethasone, imodium, oxazepam, ranitidine, enalapril, desyrel, indomethacin, sotacor, terbutaline, prednisone, glysennid, cimetidine, morphine, beclomethasone dipropionate, dimenhydrinate, ibuprofen, cyclobenzaprine, naproxen, cortisone, estazolam, imipenem, erythromycin, aspirin, metoclopramide, prochlorperazine, fursemide and hydroxymorphone. However, leritine and verapamil did produce peaks eluting close to VINC and IS. Combination of the leratine solution with VINC and IS followed by HPLC evaluation demonstrated that leratine is baseline resolved from the VINC peak and would therefore not interfere with quantitation. Verapamil, however, was not resolved from the IS peak and would therefore interfere with quantitation using the IS peak area. To characterize whether this interference from verapamil was eliminated by the plasma extraction portion of the assay procedure, plasma samples containing approximately 0.4 mg ml^{-1} of verapamil (with and without IS) were analyzed. The verapamil was not eliminated from the plasma sample during the extraction procedure and therefore represents a potential source for interference with this assay procedure for quantitation of total VINC in plasma using vinblastine as IS.

Vinblastine is well resolved from VINC using the chromatographic conditions of this assay and therefore would not interfere with analysis of

VINC. Since extraction of both VINC and vinblastine from plasma samples using the same procedure and separation with the HPLC analytical column was possible, vinblastine was selected as IS for this quantitative assay method. Vindoline (retention time 9.1 min) also elutes at a different time from both VINC (10.4 min) and IS (14.7 min) and therefore would not interfere with quantitation of VINC based on peak area.

The specificity of this assay procedure with respect to other drugs was more extensively evaluated than previously described for HPLC methods [9,11,14,15]. As anticipated, the addition of a separation step has provided specificity for VINC in the presence of vinblastine and other vinca alkaloids which is not found with many immunoassay techniques [5–7]. More recently, antibodies for VINC, generated using VINC–protein conjugates, have provided specificity for VINC [3,4] but antisera titre remains a concern and reactivity to other drugs has not been extensively evaluated.

3.9. Ruggedness

The ruggedness of the assay was estimated by the day-to-day variability in peak area ratio values obtained for four concentration values evaluated in quadruplicate on 3 days. Analysis of variance results in Table 8 demonstrate that there is statistically significant day-to-day variability in the assay procedure for the 32.7, 65.3 and 1310 ng ml^{-1} standards. The 327 ng ml^{-1} standards evaluated did not exhibit day-to-day variability (P-value 0.158). This day-to-day variability is likely due to variability in the sample handling procedure and necessitates preparing and evaluating a standard curve each day unknown samples are to be evaluated.

Table 8
Analysis of variance results for day-to-day variability

Actual concentration (ng ml^{-1})	F-observed	P-value
32.7	148	1.31×10^{-7}
65.3	59.6	1.56×10^{-5}
327	2.35	0.158
1310	28.3	0.000235

4. Conclusions

The validation of an HPLC assay procedure for determination of total VINC in plasma is described. This assay was found to be linear and is suitable for quantitation of VINC in clinical pharmacokinetic studies. The limit of quantitation was 28.6 ng ml^{-1}. Area rate precision (R.S.D. range from 3.33 to 11.6%) and accuracy of predicted values (R.S.D. range from 8.56 to 23.8%) were acceptable for quantitation of VINC in plasma. The recovery of VINC from plasma containing oncovin and VSLI ranged from 74.9 (960 ng ml^{-1}) to 88.8% (96 ng ml^{-1}) and was similar for both sources of VINC. Day-to-day variability was observed in peak area ratio values so a standard curve must be prepared and evaluated along with each set of unknown samples. Thirty-seven potentially co-administered drugs were evaluated using the HPLC assay and only verapamil represent a source for interference with quantitation of VINC as it co-elutes with vinblastine which is used as the IS. In addition, other vinca alkaloids were evaluated (vinblastine and vindoline) and will not interfere with quantitation of VINC using this procedure.

Acknowledgements

Financial support was provided by Inex Pharmaceuticals Corporation and the British Columbia Cancer Agency.

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