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A sensitive assay for the quantitative analysis of vinorelbine in mouse and human EDTA plasma by high-performance liquid chromatography coupled with electrospray tandem mass spectrometry

Carola W.N. Damen[a,*], Hilde Rosing[a], Matthijs M. Tibben[a], Maria J. van Maanen[a], Jurjen S. Lagas[b], Alfred H. Schinkel[b], Jan H.M. Schellens[c,d], Jos H. Beijnen[a,c]

[a] Department of Pharmacy and Pharmacology, Slotervaart Hospital/The Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands
[b] Department of Experimental Therapy, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[c] Beta Faculty, Department of Pharmaceutical Sciences, Division of Biomedical Analysis, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands
[d] Department of Medical Oncology, Antoni van Leeuwenhoek Hospital/The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

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ABSTRACT

A sensitive, specific and fast high-performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS) assay for the determination of vinorelbine in mouse and human plasma is presented. A 200 μL aliquot was extracted with solid-phase extraction (SPE) using Bond-Elut C_2 cartridges. Dried extracts were reconstituted in 100 μL 1 mM ammonium acetate pH 10.5–acetonitrile–methanol (21:9:70, v/v/v) containing the internal standard vintriptol (100 ng/mL) and 10 μL volumes were injected onto the HPLC system. Separation was achieved on a 50 mm × 2.0 mm i.d. Gemini C_{18} column using isocratic elution with 1 mM ammonium acetate pH 10.5–acetonitrile–methanol (21:9:70, v/v/v) at a flow rate of 0.4 mL/min. HPLC run time was only 5 min. Detection was performed using positive ion electrospray ionization followed by tandem mass spectrometry (ESI–MS/MS). The assay quantifies vinorelbine from 0.1 to 100 ng/mL using human plasma sample volumes of 200 μL. With this method vinorelbine can be measured in mouse plasma samples when these samples are diluted eight times in control human plasma. Calibration samples prepared in control human plasma can be used for the quantification of the drug. The lower limit of quantification in mouse plasma is 0.8 ng/mL. This assay is used to support preclinical and clinical pharmacologic studies with vinorelbine.

1. Introduction

Vinorelbine (Navelbine®, Fig. 1A) is a semi-synthetic vinca-alkaloid that inhibits the polymerization of tubulin dimers into microtubules, which results in the disruption of mitotic spindle formation in dividing cells [1]. The drug is mainly used in non-small cell lung cancer and breast cancer and showed promising response rates in trials with ovarian, esophageal, head and neck cancer [2]. The drug is available for both intravenous and oral administration. The absorption of oral vinorelbine is rapid (0.75–3 h) [1–4]. Vinorelbine shows a low level of binding to plasma proteins (13%) and is highly bound to platelets (78%) [3,5]. The pharmacokinetics are best characterized by a three-compartment model [6,7] and a terminal half-life of 21–41 h has been reported [1–3,7,8]. Vinorelbine is eliminated mainly via the bile [3], about 11% of the vinorelbine dose is eliminated by the kidneys [6]. Both oral and intravenous therapies are still under investigation. In these studies it is important to analyse the vinorelbine pharmacokinetics.

The role of drug transporters and cytochrome P450 systems in the pharmacokinetics of vinorelbine in mice has been investigated earlier by us [9]. To support the pharmacokinetic studies in humans and in mice a fast and sensitive bioanalytical assay suitable for plasma of both species is indispensable.

Several methods have been developed in recent years for quantification of vinorelbine in mouse, rabbit and human samples. The first method was a radioimmunoassay [10]. Later several high-performance liquid chromatographic (HPLC) methods were developed using either ultraviolet (UV) [11–13], electrochemical [14–16] or fluorescence [17–21] detection. Not only vinorelbine but also the metabolites vinorelbine-*N*-oxide [4,22,23], 4-*O*-deacetyl vinorelbine [2,4,11–13,17,20,22,23] and 20′-hydroxyvinorelbine [23] were quantified. Only 4-*O*-deacetylvinorelbine shows pharmacological activity [24]. In two papers whole blood instead of plasma was used as bioanalytical matrix [13,23]. According to the

* Corresponding author. Tel.: +31 20 5125008; fax: +31 20 5124753.
E-mail address: carola.damen@slz.nl (C.W.N. Damen).

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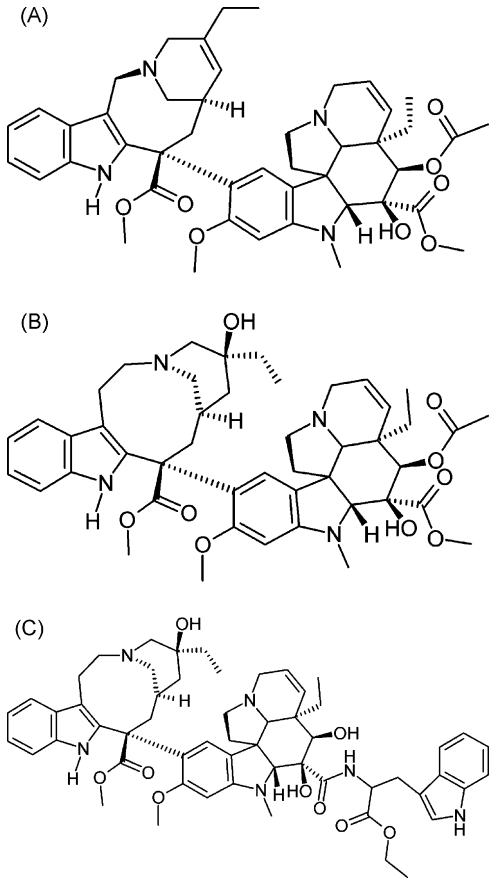

Fig. 1. Chemical structures of vinorelbine (A), vinblastine (B) and vintriptol (C).

authors the concentration of vinorelbine in blood will remain constant in contrast to the concentration in plasma when different in centrifuge conditions are used. However, vinorelbine is bound to blood platelets for 78% and the free drug concentration is closely related to the pharmacologic or toxicologic response [5]. Thus it is more important to measure the free drug concentration in plasma then the total drug concentration in whole blood. It only has to be taken into account that the sampling conditions and the centrifugation process used to separate blood cells from plasma are standardized.

Due to high sensitivity and selectivity liquid chromatography coupled online with triple quadrupole tandem mass spectrometry (LC–MS/MS) has become the first choice for the analysis of drugs in biological matrices. To date only one LC–MS [22] and one LC–MS/MS [23] method for the quantitative determination of vinorelbine in biological matrices have been described. These methods, however, have HPLC run times of 15 and 20 min, respectively, which is disadvantageous for high-throughput analysis. In these assays the analytes are chromatographically separated from matrix components using an acidic mobile phase. A basic mobile phase in combination with positive ionization is well suited for the sensitive bioanalysis of weakly basic compounds as shown earlier in our department for paclitaxel, ABT-518, elacridar and rivastigmine [25–29].

The aim of the present study was to design a fast, simple and sensitive method for the quantification of vinorelbine in human and mouse plasma using liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS). As all (pre)clinical samples will be provided in EDTA plasma, this type of plasma was chosen for method development and validation. As no stable isotopically labeled internal standard could be obtained, the vinca-alkaloids vinblastine (Fig. 1B) and vintriptol (Fig. 1C) were tested.

Table 1
Settings of the Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer

Parameter	Setting
Run duration (min)	5
Ionspray voltage (kV)	+3
Sheath gas (N_2) (psi)	49
Auxiliary gas (N_2) (psi)	19
Ion sweep gas (N_2) (psi)	5
Source CID collision energy (V)	12
Capillary temperature (°C)	400
Collision pressure (argon) (mTorr)	1.0
Chrom filter peak width (s)	10
Quad MS/MS bias (V)	2.9

	Vinorelbine	Vinblastine	Vintriptol
Q1 mass (amu)	779	811	970
Q3 mass (amu)	122	224	355
Dwell time (ms)	200	200	200
Collision energy (V)	43	44	48
Tube lens voltage (V)	145	175	198

Using a basic mobile phase in combination with positive ionization, a method was developed which is sixfold more sensitive for vinorelbine in human plasma compared to the LC–MS/MS method that has been published previously [23], using a sample volume of only 200 μL. Our method is fully validated according to the FDA guidelines on bioanalytical method validation [30,31].

2. Experimental

2.1. Chemicals and materials

Vinorelbine ditartrate ($C_{45}H_{54}N_4O_8{\cdot}2C_4H_6O_6$, potency 72.2%) and vinblastine sulphate ($C_{46}H_{58}N_4O_9{\cdot}H_2SO_4$, potency 86.5%) were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Vintriptol mesylate ($C_{56}H_{68}N_6O_9{\cdot}CH_3SO_3H$, formulated product) originated from Medgenix Group (Brussels, Belgium). HPLC grade methanol and acetonitrile were purchased from Biosolve Ltd. (Amsterdam, The Netherlands). Ammonium acetate and ammonia 25% were purchased from Merck (Darmstadt, Germany). Double distilled water was used throughout analysis. Drug-free human EDTA plasma was obtained from Bioreclamation (Hicksville, NY, USA). Drug-free mouse plasma originated from the Netherlands Cancer Institute (Amsterdam, the Netherlands).

2.2. Mass spectrometry

A Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Fisher Scientific, Waltham, MA, USA) operating in the positive ion mode was used as a detector. For quantification multiple reaction monitoring (MRM) chromatograms were acquired with LCquan™ software version 2.5 (Thermo Fisher Scientific). Positive ions were created at atmospheric pressure and the quadrupoles were operating at unit resolution (0.7 Da). Mass transitions from *m/z* 779 to 122 for vinorelbine, from *m/z* 811 to 224 for vinblastine and from *m/z* 970 to 355 for vintriptol were optimised. The ESI–MS/MS operating parameters used in this study are listed in Table 1.

2.3. Chromatographic conditions

Chromatographic separations of vinorelbine and the internal standards were carried out using a LC-20AD Prominence binary

solvent delivery system with a column oven, DGU-20A_3 online degasser and a SiL-HTc controller (Shimadzu, Kyoto, Japan). The mobile phase consisted of 1 mM ammonium acetate pH 10.5–acetonitrile–methanol (21:9:70, v/v/v) and was delivered at a flow rate of 0.4 mL/min through a Gemini C_{18} column (50 mm × 2.0 mm i.d., particle size 5 μm; Phenomenex, Torrance, CA, USA) protected with a SecurityGuard column (4 mm × 2.0 mm i.d., particle size 5 μm; Phenomenex), and thermostatted at 40 °C. The run time was 5 min. Sample injections of 10 μL were carried out and the autosampler temperature was set at 7 °C.

2.4. Preparation of stock and working solutions

Two sets of stock solutions for vinorelbine in methanol were prepared from two independent weighings at a target concentration of 1 mg/mL. One stock solution was used to prepare calibration standards, the other to prepare quality control (QC) samples. The stock solutions were further diluted with control human EDTA plasma to obtain working solutions in a range from 2 to 10,000 ng/mL for the calibration standards. For the preparation of QC samples a working solution in control human EDTA plasma at a concentration of 10,000 ng/mL was prepared. The working solutions were prepared in control human EDTA plasma to prevent the addition of large volumes of methanol to spike the calibration standards and QC samples, and thus to simulate a real sample as much as possible.

Separate stock solutions of vinblastine and vintriptol were prepared in methanol at a concentration of 1 mg/mL. Internal standard working solutions of 1000 ng/mL vinblastine and vintriptol in methanol were prepared from the stock solution. For vintriptol an additional working solution of 100 ng/mL was prepared in reconstitution solvent (1 mM ammonium acetate pH 10.5–acetonitrile–methanol (21:9:70, v/v/v). The plasma working solutions were used immediately after preparation. The stock and working solutions in methanol and reconstitution solvent were stored at nominally −20 °C until use.

2.5. Preparation of calibration standards and QC samples in plasma

Before use, control human or mouse EDTA plasma was centrifuged for approximately 10 min at 3900 × *g*. Calibration standards were prepared freshly from the plasma working solutions in human EDTA plasma at concentrations of 0.1, 0.5, 1, 5, 25, 50, 75 and 100 ng/mL vinorelbine, and vortex-mixed for approximately 30 s before processing. Standards were processed and analysed in duplicate. QC samples were spiked separately to control human EDTA plasma at concentrations of 0.1, 0.3, 5, 80, 100 and 500 ng/mL by diluting the working solution in volumetric flasks.

The vinorelbine concentrations in mice were expected to be above the upper limit of quantification [9]. Therefore mouse plasma samples were diluted eight times. As mouse plasma is hard to obtain we diluted the mouse samples with drug-free human EDTA plasma before analysis. To validate if this procedure was possible and if it was possible to analyse these samples on a calibration curve in human EDTA plasma, QC samples at concentrations of 0.3, 5 and 80 ng/mL vinorelbine were prepared containing 12.5% mouse plasma. All QC samples were stored at nominally −20 °C.

2.6. Extraction procedure

Vinorelbine and the internal standard vinblastine were isolated from plasma using solid-phase extraction (SPE). To 200 μL plasma sample aliquots, 10 μL of vinblastine working solution (1000 ng/mL) was added. The samples were vortex-mixed for approximately 10 s and centrifuged for 10 min at 10,500 × *g*. The Bond-Elut C_2 cartridges (1 mL/100 mg, Varian, Harbour City, CA, USA) were activated with two times 1 mL of methanol and two times 1 mL of water. A 200 μL aliquot of plasma sample was loaded onto the cartridge. After washing the column with two times 1 mL water, the cartridge was dried for 1 min under maximum vacuum. The analytes were eluted with 1 mL of methanol and the solvent was evaporated under a gentle stream of nitrogen at 40 °C. The residue was dissolved in 100 μL of vintriptol internal standard working solution (100 ng/mL in 1 mM ammonium acetate pH 10.5–acetonitrile–methanol (21:9:70, v/v/v)) by vortex mixing for 1 min. The samples were centrifuged for 10 min at 10,900 × *g* and the clean supernatant was transferred to a glass autosampler vial with insert.

2.7. Validation procedures

A full validation of the assay in human EDTA plasma was performed according to the FDA guidelines including linearity, inaccuracy, precision, specificity, selectivity, cross-analyte/internal standard interference, ion suppression, recovery, carry-over and stability [30,31]. In 12.5% mouse plasma inaccuracy and precision were determined.

Eight non-zero plasma calibration samples were prepared and analysed in duplicate in three separate analytical runs. The linear regression of the ratio of the areas of the analyte and internal standard peaks versus the concentrations were weighted. In order to establish the best weighing factor the back-calculated calibration concentration was determined. The model with the lowest total bias and most constant bias across the range was considered the best fit. The linearity was evaluated by means of back-calculated concentrations of the calibration standards. The deviations from the nominal concentrations should be within ±20% for the lower limit of quantification (LLOQ) and within ±15% for the other concentrations with coefficient of variation (CV) values less than 20% and 15% for both the LLOQ and the other concentrations, respectively [30,31].

Inaccuracy and precision of the assay were established by analysing six replicates of QC samples of vinorelbine together with a complete set of calibration standards in three analytical runs. Samples with vinorelbine concentrations above the upper limit of quantification (ULOQ) of the calibration curve were analysed after dilution in control human EDTA plasma. Six replicates of each sample were diluted 10 times and were analysed in one analytical run. Intra-assay inaccuracy was determined as the percent difference between the mean concentration per analytical run and the nominal concentration, inter-assay inaccuracy as the percent difference between the mean concentration after three analytical runs and the nominal concentration. The coefficient of variation (CV%) represents the measure of intra- and inter-assay precision. Inaccuracy should be within ±15% except at the LLOQ concentration, where it should be within ±20%. Precisions CV% should be less than 15% except at the LLOQ concentration, where it should be less than 20% [30,31].

To investigate whether endogenous matrix constituents interfered with the assay, six individual batches of control drug-free human EDTA plasma samples containing neither analyte nor internal standards (double blank), samples containing only internal standard (blank), and LLOQ samples were prepared. Samples were processed according to the described procedures and analysed.

Additionally, to investigate whether there is cross-interference between vinorelbine and the internal standards, an interference check was performed. Control drug-free human EDTA plasma was spiked at ULOQ level and was processed and analysed without internal standards. Also drug-free plasma with only internal standard vinblastine and with only internal standard vintriptol was pro-

cessed and analysed. The response of any interfering peak with the same retention time as vinorelbine should be less than 20% of the response of a LLOQ standard. The response of any interfering peak with the same retention time as the internal standards vinblastine and vintriptol should be less than 5% of the response of the internal standards. Deviations from the nominal concentrations should be within ±20% [30,31].

For the determination of ion suppression, control drug-free human EDTA plasma was processed and dry extracts were dissolved in solutions that represent 100% recovery containing the analyte (at concentrations of 0.6, 10 and 160 ng/mL vinorelbine) and internal standards (at concentrations of 100 ng/mL for both vinblastine and vintriptol) in reconstitution solvent. Ion suppression (matrix effect) was determined by comparing the analytical response of these samples to that of the solutions containing only analyte and internal standards in reconstitution solvent at the same concentrations as mentioned above. The loss in signal represents the ion suppression [32,33].

SPE recovery of vinorelbine and internal standard vinblastine was determined by comparing the analytical response of processed QC samples (at the concentrations of 0.3, 5, 80 ng/mL for vinorelbine and 50 ng/mL for vinblastine) with the analytical response of blank samples reconstituted with solutions as described in the previous section. These experiments were performed in triplicate. Overall recovery was determined by comparing the analytical response of SPE processed QC samples with the analytical response of the samples containing only analyte and internal standards in reconstitution solvent.

Carry-over was tested by injecting two processed blank matrix samples sequentially after injecting an ULOQ sample. The response in the first blank matrix at the retention times of vinorelbine, vinblastine and vintriptol should be less than 20% of the mean response of a LLOQ sample.

To validate the method for mouse plasma, spiked QC samples (0.3, 5 and 80 ng/mL in 12.5% mouse plasma) were analysed in triplicate using calibration standards prepared in human EDTA plasma. Intra-assay inaccuracy should be within 15% and intra-assay precision CV% should not exceed 15% [30,31].

The stability of vinorelbine in spiked human EDTA plasma samples after three freeze/thaw cycles from nominally −20 °C to ambient temperatures was investigated in sixtiplicate by comparing QC samples that had been frozen and thawed three times with the initial concentrations. The stability of vinorelbine in spiked human EDTA plasma maintained at ambient temperatures for 24 h was evaluated in sixtiplicate and compared to QC samples that had remained at −20 °C. The stability of vinorelbine in spiked human EDTA plasma stored at −20 °C was evaluated triplicate and compared with the nominal concentrations. Unfortunately the initial concentration is not know in this particular case as the samples were not analysed immediately after preparation. Additionally, the stability of the dry extracts (triplicate) and in the reconstituted extracts (sixtiplicate) were determined at 2–8 °C and compared with the initial concentrations. The re-injection reproducibility in the autosampler was determined in triplicate after 24 h and compared with the initial concentrations.

The above described stability experiments were executed at two concentration levels (0.3 and 80 ng/mL vinorelbine). The analytes are considered stable in the biological matrix or extracts if 85–115% of the nominal concentration is recovered. Stability of stock solutions of vinorelbine, vinblastine and vintriptol stored at ambient temperatures for 6 h and at −20 °C was assessed in triplicate. The analyte is considered stable in stock solutions if 95–105% of the initial concentration is recovered and the internal standards are considered stable if 80–120% of the initial concentration is recovered [30,31].

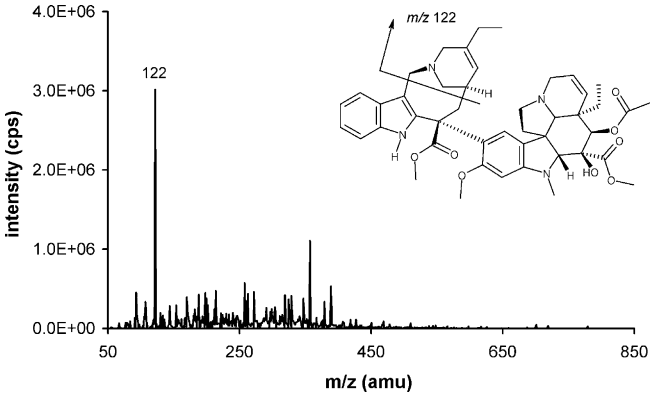

Fig. 2. MS/MS product ion scan of vinorelbine (precursor ion *m*/*z* 779).

3. Results and discussion

3.1. Mass spectrometry

During optimization of the mass spectrometric parameters, the most intense peak in the Q1 spectrum of vinorelbine corresponded to the singly charged molecular ion at *m*/*z* 779. Q1 spectra of vinblastine and vintriptol also showed the singly charged molecular ion as most intense ions at *m*/*z* 811 and 970, respectively. MS/MS experiments were performed to determine the most abundant fragment ions for multiple reaction monitoring. Figs. 2–4 show MS/MS product ions scans of vinorelbine, vinblastine and vintriptol, respectively and the proposed fragmentation reactions. The most abundant product ions were optimised using MRM (see Table 1).

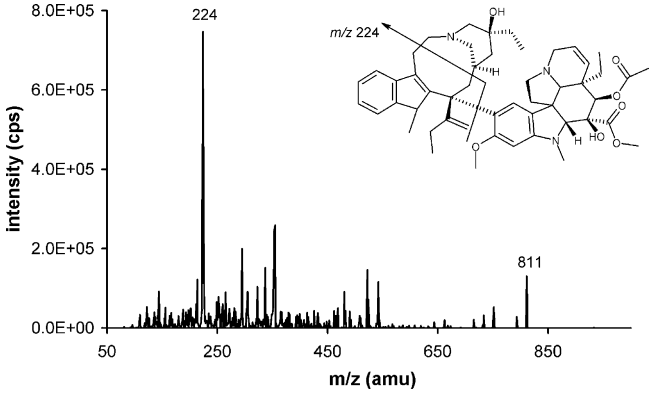

Fig. 3. MS/MS product ion scan of vinblastine (precursor ion *m*/*z* 811).

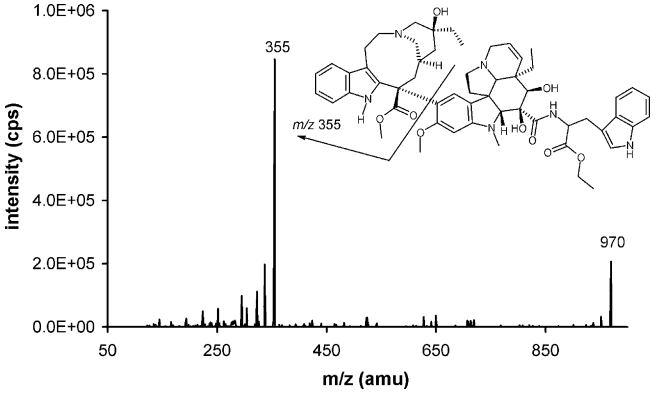

Fig. 4. MS/MS product ion scan of vintriptol (precursor ion *m*/*z* 970).

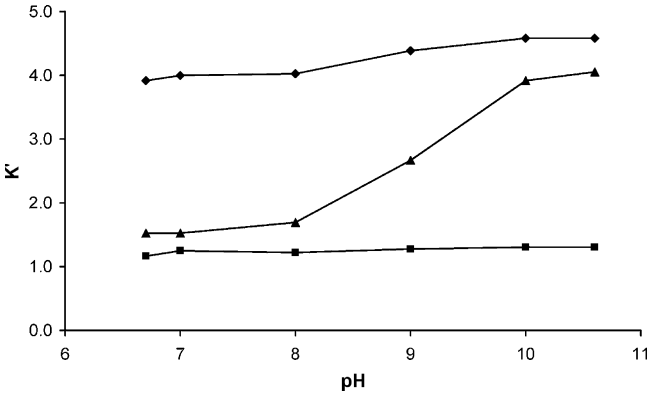

Fig. 5. Capacity factor k' versus pH for vinorelbine (▲), vinblastine (■) and vintriptol (♦). 10 mM ammonium acetate was mixed with 10 mM ammonium hydroxide to create buffers with different pH values. These buffers were mixed with methanol (30:70, v/v) and run isocratically at a flow rate of 0.4 mL/min at 40 °C.

For vinorelbine different collision pressures were tested. When 0.6 mTorr was used a fragment at m/z 323 was formed, corresponding with the catharanthine part of the molecule [34]. However the transition from m/z 779 to 122 at 1.0 mTorr provided a higher signal to noise ratio then the transition from m/z 779 to 323 at 0.6 mTorr and therefore this transition was chosen for quantification. Due to the high molecular weight of vinorelbine and the internal standards the method is very specific and as the method will be used for pharmacokinetic studies, only one transition was chosen for quantification purposes.

3.2. Chromatography

Two reports have described assays for the quantitative analysis of vinorelbine using HPLC coupled on-line with mass spectrometry [22,23]. These platforms use a cyano [23] or a reversed phase C_{18} [22] column with acidic eluents and have run times of 20 and 15 min, respectively. In order to develop a faster and more sensitive analytical system we have tested several HPLC columns: Gemini C_{18} (150 mm × 2.0 mm and 50 mm × 2.0 mm i.d., 5 μm particle size), Synergi Fusion RP C_{18} (50 mm × 2.0 mm i.d., 5 μm particle size) and Xterra C_8 (50 mm × 2.1 mm i.d., 5 μm particle size). The isocratic eluents tested were formic acid in water (pH 3), acetic acid in water (pH 4), 1 mM ammonium hydroxide (pH 9.9), 10 mM ammonium hydroxide (pH 10.6), 10 mM ammonium acetate (adjusted to pH 10 with 25% ammonia) in combination with either methanol or acetonitrile.

On the Synergi Fusion column severe tailing ($As \approx 1.5$, with a peak width at 10% height of more then 50 s) was observed with the basic eluents and with the acidic eluents vinblastine eluted as a split peak, thus this column was unsuitable for our application. The Xterra column appeared to be unsuitable as well because asymmetry factors of 2.1 were observed. The Gemini column provided the best peak shape and sufficient retention was already obtained on the column of 5 cm length, therefore this column was used for further method development. With an acidic eluent of pH 3 however, peak shapes were poor with an asymmetry factor of 3.3. By using basic eluents asymmetry factors around 1.3 were obtained for vinorelbine. In order to establish the optimal pH of the eluent 10 mM ammonium acetate was mixed with 10 mM ammonium hydroxide to create buffers in the pH range of 6.7–10.6. These buffers were mixed with methanol (30:70, v/v) and run isocratically at a flow rate of 0.4 mL/min. Fig. 5 shows the capacity factor (k') plotted against the pH. In order to retain vinorelbine on the column and to establish stable retention times a pH of 10.5 or higher should be applied. 1 and 10 mM ammonium acetate adjusted at pH 10.0, 10.5 and 11.0 with ammonia 25% and 10 mM ammonium hydroxide (pH 10.6) were compared and tested with either methanol, acetonitrile or a combination of these modifiers. Ammonium acetate provided smaller peaks than ammonium hydroxide (peak width at 10% height 19 s versus 23 s, respectively). Additionally, with 1 mM ammonium acetate the MS signal for vinorelbine is five times higher than with 10 mM ammonium acetate. At all three pH values tested with ammonium acetate, the retention times for the three compounds were exactly the same. With methanol as organic solvent moderate tailing was observed, while with acetonitrile split peaks were obtained. The best chromatographic system was realized using a mixture of methanol and acetonitrile. A small amount (9%) of acetonitrile in the eluent seems pivotal to generate excellent peak shape. HPLC column temperatures from 20 to 45 °C were tested. The higher the temperature the smaller the peak width at 10% of the height. From 40 °C no improvement in peak shape was gained anymore and therefore this temperature was chosen. In conclusion, we found that the most appropriate eluent was a mixture of 1 mM ammonium acetate pH 10.5–acetonitrile–methanol (21:9:70, v/v/v) pumped at a flow rate of 0.4 mL/min.

Representative chromatograms of vinorelbine and the internal standards vinblastine and vintriptol at the LLOQ level in human EDTA plasma are depicted in Fig. 6. Peak shapes were excellent, with asymmetry factors of 1.3 for vinorelbine and vinblastine and 1.2 for vintriptol. Signal to noise ratio (S/N) at the LLOQ level was approximately 30. The capacity factors (k') were approximately 2.7 for vinorelbine, 1.1 for vinblastine and 2.8 for vintriptol. In this system vincristine was also tested as possible internal standard, but this compound had a capacity factor of only 0.8 and was omitted from the validation. The LC run time was set at 5 min. This run time was chosen, as the noise level in the vinorelbine transition stayed high for few minutes after injection of a vinorelbine sample at ULOQ level.

3.3. Sample pre-treatment

Different methods of sample pre-treatment were investigated. As protein precipitation (PP) is by far the easiest and fastest way of sample pre-treatment, this procedure was tested. The proteins in 100 μL plasma were precipitated by adding 200 μL organic solvent. Methanol, acetonitrile and a mixture of both (methanol–acetonitrile (50:50, v/v)) were investigated as potential precipitation solvents. The total recovery of the compounds by using methanol was higher and more reproducible than with the other organic solvents and was found to be at least 91% for vinorelbine and 60% for the internal standards, therefore this sample pre-treatment was chosen for further use. Unfortunately however, after approximately 300 injections the HPLC column performance decreased dramatically. Analyte/internal standard ratios were not constant and MS signals decreased by approximately a factor 40. Therefore, protein precipitation was discarded as sample pre-treatment for this assay.

We continued by investigating liquid liquid extraction (LLE) as method for sample pre-treatment. LLE using diethyl ether is described in previous studies [11,13–15,18,19,21]. van Tellingen et al. [21] tested also chloroform, leading to very low recoveries. Additionally, Mouchard-Delmas et al. [14] investigated chloroform, chloroform–methanol mixtures and dichloromethane–methanol mixtures. Diethyl ether proved to be the best extraction solvent and a miniaturized extraction procedure was tested.

When different plasma batches were extracted with diethyl ether large batch-to-batch variations in extraction recovery and ion suppression were observed. The internal standards did not correct

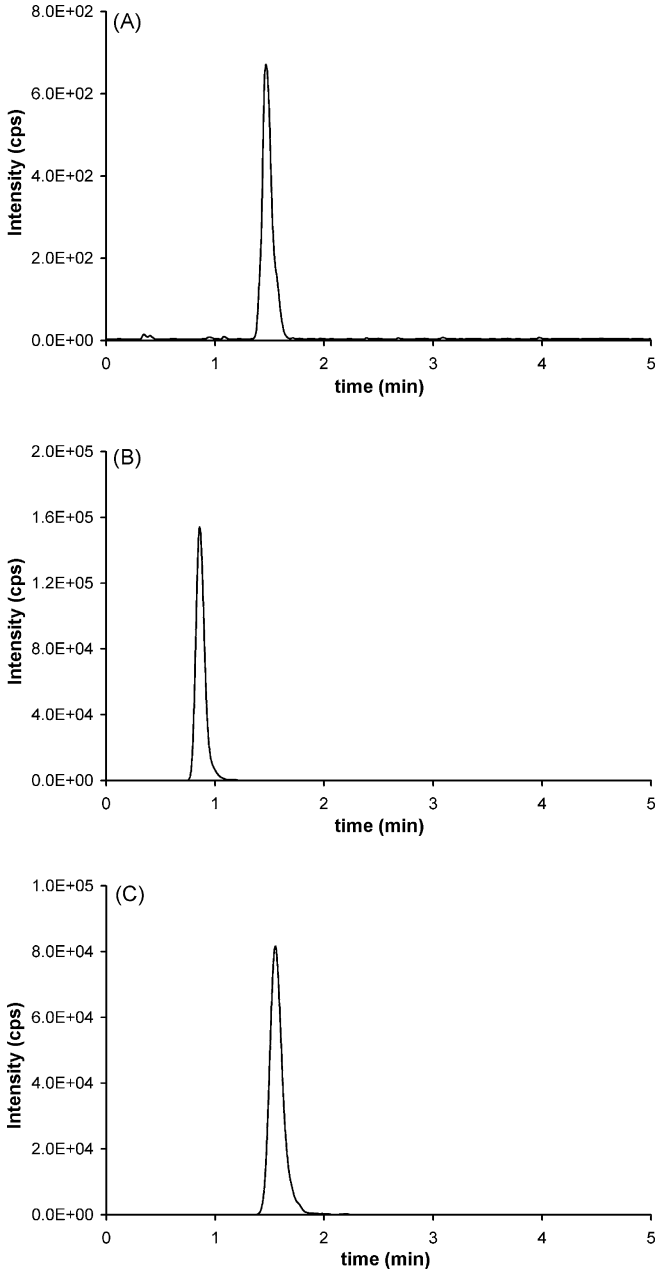

Fig. 6. Representative HPLC–MS/MS chromatograms spiked human EDTA plasma sample at the LLOQ for vinorelbine (A, 0.1 ng/mL, t_r = 1.5 min), the internal standards vinblastine (B, 50 ng/mL, t_r = 0.9 min) and vintriptol (C, 50 ng/mL, t_r = 1.6 min).

for these variations and therefore the LLE extraction was discarded as sample pre-treatment procedure.

Solid-phase extraction (SPE) for vinca alkaloids was also reported using Extrelut-3 [22], Bond-Elut CN [16] and OASIS HLB [23] columns. We investigated a wide range of columns including Bond-Elut CN-U, Bond-Elut PH, Bond-Elut C_{18}, Bond-Elut C_2, Isolute-CN, OASIS MCX, OASIS MAX and OASIS HLB. The highest recoveries were obtained with Bond-Elut C_2, Isolute-CN, OASIS MAX and OASIS HLB with acidified plasma. The most reproducible results were obtained using Bond-Elut C_2. Several plasma batches were tested and recoveries of vinorelbine and the internal standard vinblastine were very reproducible from batch to batch. Recovery of vintriptol, however, was highly variable for the tested plasma batches. Therefore, the compound was added after the SPE procedure during the reconstitution of the dried extracts. This resulted in reproducible vinorelbine/vintriptol ratios when different control plasma batches were tested.

3.4. Validation

The assay was linear over a concentration range of 0.1–100 ng/mL of vinorelbine in human plasma. The linear regression of peak area versus the concentration $1/x^2$ (the reciprocal of the squared concentration) was weighted to obtain the lowest total bias and the most constant bias across the range. When vinblastine was used as an internal standard 2 out of 3 validation runs were rejected because more then 25% of the calibration standard samples did not met the pre-defined criteria. Therefore, vinblastine was not a suitable internal standard for this assay and was omitted in further description of the validation results.

When vintriptol was used as internal standard, correlation coefficients (r^2) of 0.992 or better were obtained. At all concentration levels deviation of measured concentrations from nominal concentration were between −8.38 and 3.35% with CV values of less than 8.81%.

Assay performance data for vinorelbine in human EDTA plasma are summarised in Table 2. The intra-assay inaccuracies (%bias) for vinorelbine were within ±18.0% for the LLOQ and within ±12.1% for other concentrations. The intra-assay precisions CV% for vinorelbine were less than 11.1% for all concentrations. Samples above the ULOQ (500 ng/mL) were diluted 10 times with control drug-free human EDTA plasma. The intra-assay inaccuracy was −1.09% and the intra-assay precision CV% 2.30%. In conclusion, the validated range for vinorelbine based on 200 μL human EDTA plasma is from 0.1 to 100 ng/mL. When concentrations above 100 ng/mL are expected, plasma samples can be diluted 10 times with control drug-free human EDTA plasma. Inaccuracies and precisions fulfilled the required criteria [30,31].

MRM chromatograms of six batches of control drug-free EDTA plasma contained no co-eluting peaks >20% of the vinorelbine peak area at the LLOQ level, and no co-eluting peaks >5% of the area of the internal standard vintriptol. Deviations form the nominal concentrations at the LLOQ level were between −16.0 and 0.990% for vinorelbine and found to be acceptable [30,31].

There were no peaks detected at the retention time of vinorelbine when a sample was only processed with the internal standard vintriptol. Additionally, there were no peaks detected at the retention time of vintriptol when a vinorelbine sample at ULOQ was processed without internal standard. Thus, no cross-analyte/internal standard interference was detected.

Mean ion suppression of −14.9% (range −16.2 to −12.3%, enhancement) was detected for vinorelbine. The mean ion suppression for vintriptol was 4.79%. For vinorelbine the mean SPE recovery was 59.7% (range 53.8–65.3%) and the mean total recovery 74.6%.

In literature it is described that vinorelbine and other vinca-alkaloids absorb to different materials leading to loss of analyte and carry-over [13,23,35]. In some methods the organic phase is not completely evaporated to dryness as problems with reconstitution were experienced especially in the low concentration range [18,19,35]. During method development we also experienced carry-over of vinorelbine. When solutions in methanol were pipetted, the plungers of the pipettes had to be protected by using pipette tips with filtertips. During development of the chromatographic system, different gradient systems were tested. Unfortunately gradient elution led to carry-over of vinorelbine due to a memory effect on the HPLC column. For this reason isocratic elution was chosen and the run time was set to 5 min in order to remove all vinorelbine from the column material. Also care should be taken with the tips of the evaporation device as they may lead to carry-over as well. Cleaning the tips with ethanol should be implemented as a standard pro-

Table 2
Assay performance data for vinorelbine

Run	Nominal conc. (ng/mL)	Mean calculated conc. (ng/mL)	Inaccuracy (%deviation)	Precision (%CV)	Number of replicates
1	0.101	0.103	1.88	8.83	6
2	0.101	0.119	18.0	1.61	6
3	0.101	0.112	11.2	6.80	6
Inter-assay	0.101	0.112	11.0	8.07	18
1	0.304	0.289	−4.87	11.1	6
2	0.304	0.267	−12.1	3.22	6
3	0.304	0.292	−4.00	6.51	6
Inter-assay	0.304	0.282	−7.12	8.13	18
1	5.06	5.29	4.58	4.74	6
2	5.06	4.59	−9.35	4.76	6
3	5.06	4.36	−10.7	4.37	6
Inter-assay	5.06	4.75	−4.84	8.73	18
1	81.0	85.1	5.00	5.77	6
2	81.0	74.6	−7.88	6.56	6
3	81.0	78.2	−3.52	8.04	6
Inter-assay	81.0	79.3	−2.13	8.52	18
1	101	102	1.01	2.20	6
2	101	93.3	−7.67	5.62	6
3	101	103	2.38	4.53	6
Inter-assay	101	99.6	−1.43	6.14	18

Conc.: concentration; CV: coefficient of variation.

cedure before inserting new samples in the evaporation device to prevent carry-over. In the present system we experienced no carry-over and to confirm this a carry-over test was performed during the validation procedures. No interfering peaks were detected in processed blank plasma samples injected after an ULOQ sample.

When samples containing 12.5% mouse plasma were quantified on calibration curves in human EDTA plasma the intra-assay inaccuracies for vinorelbine were within ±8.50% for all concentrations. The intra-assay precisions CV% for vinorelbine were less than 6.02% for all concentrations. Therefore it can be concluded that mouse plasma can be diluted eight times with control human EDTA plasma and that the samples can be analysed on calibration curves in human EDTA plasma.

The stability data for vinorelbine are presented in Table 3. All stability experiments met the predefined criteria as described in the experimental section. Thus we could conclude that vinorelbine is

Table 3
Stability data for vinorelbine and the internal standards vinblastine and vintriptol

Matrix	Conditions	Initial conc. (ng/mL)	Found conc. (ng/mL)	Dev. (%)	CV (%)	Number of replicates
Vinorelbine						
Plasma	Three freeze (−20 °C)/thaw cycles	0.302	0.262	−8.16	3.35	6
		85.1	74.6	−12.2	4.43	6
Plasma	Ambient, 24 h	0.271	0.268	−1.23	6.46	6
		83.2	77.7	−6.63	6.68	6
Plasma	−20 °C, 10 months	0.304[a]	0.259	−14.6	0.272	3
		81.0[a]	73.9	−8.72	4.56	3
Dry extract	2–8 °C, 8 days	0.283	0.295	3.94	0.240	3
		75.9	75.4	−0.703	2.41	3
Final extract	2–8 °C, 7 days	0.267	0.263	1.88	4.46	6
		68.7	75.0	6.57	5.74	6
Final extract	Autosampler, 7 °C, 24 h	0.278	0.243	−6.65	0.272	3
		5.14	4.48	−12.8	1.15	3
		84.2	76.3	−9.39	0.843	3
MeOH (stock solution)	Ambient, 6 h	1.01×10^6	1.00×10^6	−0.615	1.09	3
MeOH (stock solution)	−20 °C, 1 year	1.01×10^6	1.00×10^6	−0.797	9.08	3
Vinblastine						
MeOH (stock solution)	Ambient, 6 h	1.01×10^6	8.41×10^5	−16.8	2.98	3
MeOH (stock solution)	−20 °C, 10 months	1.01×10^6	1.06×10^6	−5.01	4.67	3
Vintriptol						
MeOH (stock solution)	Ambient, 6 h	9.94×10^5	8.65×10^5	−13.0	4.54	3
MeOH (stock solution)	−20 °C, 1 year	9.94×10^5	1.06×10^6	6.42	8.07	3
MeOH (working solution W1000)	−20 °C, 7 months	994	959	−3.52	0.904	3
Reconstitution solvent (working solution W100)	−20 °C, 4 months	99.4	81.0	−18.5	0.229	3

Conc.: concentration; Dev.: deviation; CV: coefficient of variation.
[a] Long-term stability data in plasma were compared with nominal concentrations as the initial concentration was not analysed immediately after preparation.

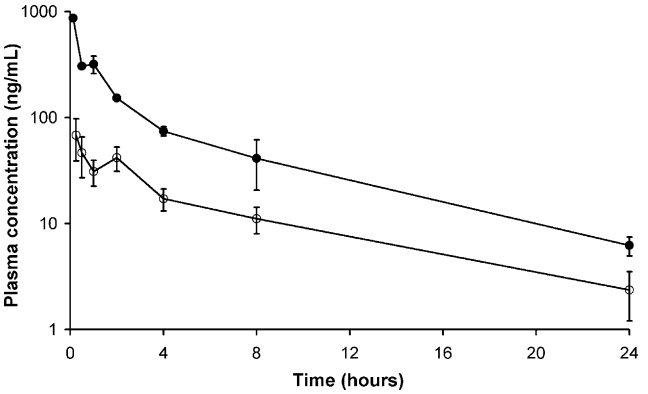

Fig. 7. Concentration versus time profile of vinorelbine in wild-type mice treated orally (○) or intravenously (●) with 10 mg/kg vinorelbine. Every point represents the average value of four mice. Error bars indicate standard deviations.

stable in human EDTA plasma for at least three freeze (−20 °C)/thaw cycles, and in human EDTA plasma at ambient temperatures for up to 6 h. Furthermore, vinorelbine is stable up to 8 days in the dry extract and up to 7 days in the final extract at nominally 2–8 °C. Re-injection reproducibility was established and the analytical run can be re-injected after at least 24 h of storage in the autosampler at 7 °C. Vinorelbine is stable in human EDTA plasma up to 10 months at −20 °C.

Stock solutions of vinorelbine, vinblastine and vintriptol are stable up to 1 year, 10 months and 1 year, respectively, when stored at −20 °C and are stable at ambient temperatures for at least 6 h. Vintriptol working solution of 1000 ng/mL in methanol is stable for at least 7 months when stored at −20 °C and vintriptol working solution of 100 ng/mL in reconstitution solvent is stable for at least 4 months when stored at −20 °C.

4. Application of the method

The validated vinorelbine assay was used to support a pharmacokinetic study in mice. Fig. 7 shows the results of wild-type FVB mice receiving 10 mg/kg vinorelbine either orally or intravenously. At different time-points whole blood was taken from the tail vein (orally) or with cardiac puncture (intravenously). Because high concentrations were expected, the mouse plasma was diluted eight times with human control EDTA plasma (25 μL mouse plasma with 175 μL human EDTA plasma) before processing. Even 24 h after administration, vinorelbine can be quantified although the plasma had been diluted eight times. These results demonstrate the applicability of the method to support (pre-)clinical pharmacokinetic studies.

5. Conclusion

In this paper the development, validation and application of a LC–MS/MS method for the quantitative analysis of vinorelbine is described. Vinorelbine is extracted from human EDTA plasma or diluted mouse plasma using solid-phase extraction followed by reconstitution with the internal standard vintriptol. Although the addition of the internal standard after the solid-phase extraction is not preferable, validation results show that the method is accurate, precise and reproducible. Chromatography is performed under alkaline conditions. In human plasma a linear dynamic range from 0.1 to 100 ng/mL was validated. The lower limit of quantification for mouse plasma was 0.8 ng/mL when mouse plasma was diluted eight times in control human plasma. The method is easy to perform and fast, and it has demonstrated its applicability in pre-clinical and clinical pharmacologic research.

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