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Validation of an electrospray ionization LC/MS/MS method for quantitative analysis of vincristine in human plasma samples

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ABSTRACT

Vincristine is a natural vinca alkaloid widely used in paediatric cancer treatment. Vincristine pharmacokinetics has been already studied, but few data are available in paediatric populations. A sensitive and specific liquid chromatography–tandem mass spectrometry (LC/MS/MS) method was developed for the quantification of vincristine in plasma in order to investigate pharmacokinetics in a paediatric population. Two hundred microliters of plasma was added to vinblastine, used as internal standard. Chromatographic separation was achieved on a C8 HPLC column (Phenomenex Luna 50 mm × 2.0 mm, 3.0 μm) with a mobile phase gradient at a flow rate of 0.2 ml/min. Quantification was performed using the transition of 825.4 → 765.4 (*m*/*z*) for vincristine and 811.4 → 751.4 (*m*/*z*) for vinblastine. Chromatographic separation was achieved in 8 min. The limit of quantification was 0.25 ng/ml with a precision of 10.2% and an accuracy of 99.6%. The calibration curve was linear up to 50.0 ng/ml. Intra-day precision and accuracy ranged from 6.3% to 10% and from 91.9% to 100.8%, respectively. Inter-assay precision and accuracy ranged from 3.8% to 9.7% and from 93.5% to 100.5%, respectively. No significant matrix effect was observed for vincristine. A rapid, specific and sensitive LC/MS/MS method for quantification of vincristine in human plasma was developed and is now successfully applied for pharmacokinetic studies in paediatric patients.

1. Introduction

Vincristine is an antimicrotubule agent belonging to the vinca alkaloids, derived from *Catharanthus roseus*, and has been used for the treatment of different human neoplastic disorders for nearly 40 years. Vincristine plays a pivotal role in the treatment of paediatric acute leukaemia, but it is also used in the management of Hodgkin's and non-Hodgkin's lymphomas as well as in solid tumors such as rhabdomyosarcoma, neuroblastoma, Wilms tumor, and multiple myeloma [1]. Although vincristine has been used since the 1960s, the clinical pharmacology of vincristine remains an open question. Indeed, vincristine pharmacokinetics is characterized by great inter-individual variability [2,3]. However, the impact on clinical outcome (e.g. toxicity and survival) is not well understood.

Consequently, an appropriate method for the quantification of vincristine in plasma is required for pharmacokinetic studies, in particular for the paediatric population, for which a high sensitivity with a small plasma sample volume is required. Radioimmunoassay methods were first developed to quantify vincristine in human samples, but they were characterized by a lack of specificity between the parent drug and metabolites [4]. Conventional high-performance liquid chromatography (HPLC) methods with UV and fluorimetric detection have failed to reach adequate sensitivity [5]. In the last decade, liquid chromatography tandem mass spectrometry (LC/MS/MS) became the reference method owing to its high specificity, sensitivity and reliability. Recently, different LC/MS/MS methods for vincristine quantification have been reported, but all of them use a large plasma sample (500 μl) [6–8]. Only Corona et al. [9] determined plasma vincristine concentrations using LC/MS/MS with a smaller plasma sample (100 μl). However, this method was based on an on-line extraction step after precipitation, which requires additional instruments.

Our aim was to develop and validate a sensitive, specific and rapid LC/MS/MS method for the quantification of vincristine in plasma that would be suitable for pharmacokinetic studies in paediatric populations.

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Table 1
Gradient elution for chromatographic conditions.

Time (min)	% Phase A	% Phase B	Flow (ml/mn)	Gradient
0.0	70	30	0.2	None
4.0	0.0	100	0.2	Linear
4.1	70	30	0.2	None
8.0	70	30	0.2	None

2. Materials and methods

2.1. Chemicals and reagents

Vincristine and vinblastine, used as internal standard (IS) for this assay, were obtained from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile HPLC grade and ammonium acetate were purchased from VWR Prolabo (Fontenay-sous-Bois, France). Methanol LC/MS hypergrade was provided by Merck Research Laboratories (Rahway, NJ, USA). HPLC grade water was produced with a Milli-Q® academic system (Millipore, Bedford, MA, USA). Acetic acid and ammonium acetate were obtained from VWR Prolabo (Fontenay sous Bois, France). Blank plasma from healthy donors was kindly supplied by the French Blood Bank ("Etablissement Français du Sang", EFS, Marseille, France).

2.2. Chromatographic conditions

A Waters Alliance 2795 HPLC separation module (Waters, Milford, MA, USA) with cooled autosampler and column oven was used to perform this assay. Chromatographic separation was achieved on a Luna C8 50 mm × 2.0 mm, 3.0 μm column (Phenomenex, Torrance, CA, USA), maintained at 50 °C. The mobile phases consisted of acetic acid 1% in water titrated to pH 4 with ammonia (mobile phase A) and acetonitrile (mobile phase B). The gradient elution was performed at a flow rate of 0.2 ml/min (Table 1).

2.3. Mass spectrometry conditions

A tandem quadrupole MS Quattro Premier XE (Waters, Milford, MA, USA), operated in multiple reaction monitoring in positive electrospray ionisation mode, was used for detection and Masslynx 4.1 software was used for data acquisition and processing. MS/MS detection was conducted by monitoring the fragmentation of 825.4 → 765.4 (*m*/*z*) for vincristine and 811.4 → 751.4 (*m*/*z*) for vinblastine. The instrument response was optimized by syringe pump infusion of vincristine and vinblastine in mobile phase by constant flow (20 μl/mn) into the stream of mobile phase eluting from the LC column.

Source parameters were as follows: capillary voltage, 3.00 kV; cone voltage, 60 V and 65 V for vincristine and vinblastine, respectively; extractor voltage, 3.00 kV; RL lens voltage, 0 V; source temperature, 120 °C; desolvatation temperature, 350 °C; cone gas flow, 50 l/h; desolvatation gas flow, 800 l/h.

Analysis parameters were: LM 1 resolution, 14.5; HM 1 resolution, 15.0; ion energy, 0.2 eV; entrance energy, 1 eV; collision energy, 45 eV and 40 eV for vincristine and vinblastine, respectively; exit energy, 0 eV; LM 2 resolution, 13.5; HM 2 resolution, 13.5; ion energy 2, 0.5 eV; multiplier voltage, 650 V.

Detection conditions were: inter channel delay, 0.02 s; span, 0.2 Da; dwell time, 0.2 s; start time, 0.0 min; end time, 8.0 min.

2.4. Preparation of stock solutions, calibration standards and quality control samples

Vincristine and IS stock solutions were prepared in a solution of methanol/HPLC grade water (1:1, v/v) to obtain a final concentration of 1 mg/ml. Aliquots of stock solutions were then stored at −40 °C for vincristine and IS for no more than 12 months. Vincristine and IS stock solutions were further diluted in methanol/HPLC grade water (1:1, v/v) on the day of the extraction, to obtain respectively working solutions with concentrations of 1, 0.1, 0.01 and 0.001 μg/ml for vincristine and 0.1 μg/ml for vinblastine. Calibration standards were prepared, by dilution of various volumes of the working solution in blank human plasma, at 0.25, 0.5, 1, 2.5, 5, 10, 25 and 50 ng/ml. Aliquots of calibration standards were stored at −40 °C.

Quality control (QC) samples containing vincristine were obtained with a different vincristine stock solution and prepared in blank plasma at concentrations of 0.5, 2.5, 7.5 and 30 ng/ml. Aliquots of the QC samples were kept at −40 °C.

2.5. Sample processing

The developed extraction method was based on a solid-liquid extraction. The solid phase extraction consisted of an Oasis HLB 1 ml/10 mg extraction cartridge (Waters, Milford, MA, USA), conditioned with 400 μl of methanol followed by 400 μl of water. Two hundred microliters of spiked plasma was mixed with 20 μl of 0.1 ng/ml IS solution, vortexed and loaded onto the column. The column was washed twice with 400 μl of 5% methanol in water and vincristine was eluted with 400 μl of 100% methanol. The extracted samples were evaporated and reconstituted in 100 μl of mobile phase A/acetonitrile (7:3, v/v), then transferred into 96-well plates (Waters®). Twenty microliters was injected into the column for analysis.

2.6. Validation procedures

Validation of the assay was performed in accordance with FDA guidelines for Bioanalytical Methods Validation for Human Studies [10].

2.6.1. Linearity and sensitivity

Calibration standards were prepared and analyzed in 6 replicates in 6 independent runs. Calibration curves were fitted using the linear regression. In order to obtain acceptable linearity, deviations of the mean calculated concentrations over 6 runs had to be within ± 15% of nominal concentrations for the non-zero calibration standards, except for the limit of quantification (LOQ) level where a deviation of ±20% was allowed [10]. The LOQ was determined by analyzing 6 replicates in the same run. A precision of ±20% and an accuracy of 80–120% were allowed for the LOQ [10].

2.6.2. Accuracy and precision

Intra-assay precision and accuracy were determined by analyzing 6 replicates of each spiked QC sample on a single assay. Inter-assay precision and accuracy were determined by analyzing one QC sample per day at each concentration for 6 different days. Intra- and inter-assay precisions were expressed as the coefficient of variation (CV) at each QC concentration and could not exceed ±15% [10]. Accuracy was calculated as the percent deviation from the nominal concentration and had to be within ±15% [10].

2.6.3. Extraction recovery

Extraction recovery of the analyte at the four QC levels was determined by comparing peak areas, obtained from extract plasma samples (in triplicate), with those found by direct injection of diluted stock solution at the same concentration.

2.6.4. Matrix effect

Matrix effect was evaluated on 6 different extracts of blank plasma samples spiked at 25 ng/ml injected in duplicate. We

compared peak area obtained from the plasma extract with the peak area obtained from the diluted stock solution at the same concentration made in the reconstitution reagent (mobile phase A/acetonitrile (7:3, v/v)).

2.6.5. Selectivity and specificity

Interferences from endogenous plasma compounds were investigated by analyzing 6 plasma samples from paediatric patients treated for their tumor diseases with different antineoplasic agents (actinomycine, cyclophosphamide, irinotecan), except vincristine.

2.6.6. Stability

Long-term stability was assessed after storage of six patient samples at −80 °C for 6 months. Freeze/thaw stability was determined after three freeze/thaw cycles (−80 °C to room temperature), in six patient plasma samples. Extract stability at +4 °C was determined during 48 h at the four QC levels. The analyte was considered stable in the matrix when the concentration difference between the fresh sample and the stability testing sample did not exceed 20%.

2.7. Method application

Vincristine concentrations were determined in plasma samples using the above described method. Blood samples were drawn in six children (7.5–13 years) treated for Ewing sarcoma as part of a study approved by the institutional review board of the Timone Hospital (Marseille, France). A mean dose of $1.2 \pm 0.36\ mg/m^2$ of vincristine was administered and plasma samples were collected following a sparse sample strategy (from 10 min to 24 h). Samples were centrifuged, separated and stored at −80 °C until analysis.

3. Results and discussion

Vincristine and its IS were detected and quantified over a total run time of 8 min. All analytes were optimized prior to their quantification by LC/MS/MS. For each selected transition of vincristine (m/z 825.4 → 765.4) and IS (m/z 811.4 → 751.4), retention times were 2.61 min and 2.80 min, respectively. Representative chromatograms of an extracted blank plasma sample and extracted plasma enriched with vincristine at 0.25 ng/ml are shown in Fig. 1.

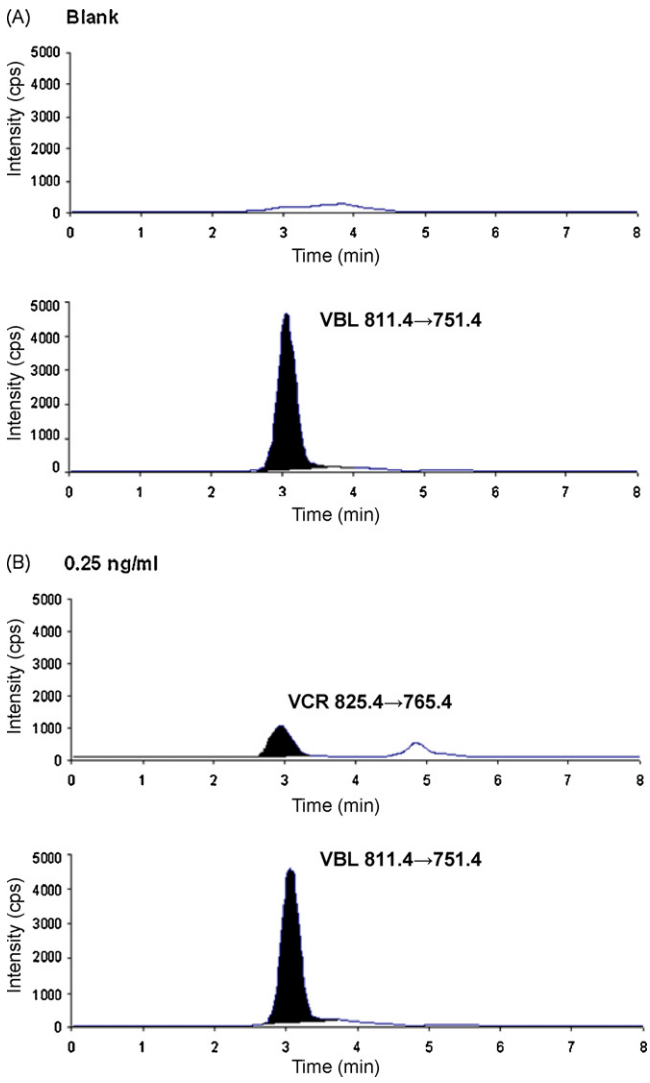

Fig. 1. Typical ion chromatogram of vincristine and IS (vinblastine) obtained from a blank sample (A) and a blank sample enriched with vincristine at 0.25 ng/ml (B).

3.1. Validation of the analytical method

3.1.1. Linearity and sensitivity

The assay was linear over the range 0.25–50 ng/ml, and the linear regression analysis yielded the following equation (95% Confidence Interval): $y = 0.0791(0.0772 - 0.0810)x + 0.00165(-0.0368 - 0.0401)$ ($r^2 = 0.998$). A reproducible linear relationship between concentration and response was found over the measured concentration range. Moreover, a precision ranging from 7.05% to 13.8% and a deviation from −4.1% to 6.79% were observed across the 8 calibration levels (6 replicates). Mean calculated concentrations over 6 runs did not deviate by more than ±15% from nominal concentrations for the non-zero calibration standards. The limit of quantification (LOQ) was established to 0.25 ng/ml with a precision of 10.2% and an accuracy of 99.6%, based on a signal-to-noise ratio of 5. Therefore, the response at LOQ was identifiable, discrete and reproducible with a precision of ±20% and an accuracy of 80–120% according to the FDA guidelines [10].

3.1.2. Precision and accuracy

The results of assay performance, assessed at four QC concentrations, are summarized in Table 2. Intra-day precision and accuracy ranged from 6.3% to 10% and from 91.9% to 100.8%, respectively. Inter-assay precision and accuracy ranged from 3.8% to 9.7% and from 93.5% to 100.5%, respectively. All observed data for the intra- and inter-assay were below 10%. These data indicated that the method provides adequate accuracy and precision for vincristine determination in plasma samples.

Table 2
Intra-day and inter-day precision and accuracy for vincristine ($n = 6$).

Vincristine (ng/ml)	Intra-day				Inter-day			
	0.5	2.5	7.5	30	0.5	2.5	7.5	30
Mean (ng/ml)	0.480	2.30	6.99	30.2	0.502	2.34	7.26	29.9
SD (ng/ml)	0.0315	0.144	0.509	3.01	0.0189	0.115	0.576	2.81
Precision (CV%)	6.6	6.3	7.3	10.0	3.8	4.9	7.9	9.4
Accuracy (%)	−4.0	−8.1	−6.9	0.79	0.47	−6.5	−3.2	−0.50

Table 3
Recovery of vincristine following solid/liquid extraction.

Vincristine (ng/ml)	*n*	Mean extraction recovery (%)	CV (%)
0.5	3	67	11
2.5	3	64	12
7.5	3	73	9.2
30	3	78	5.8

3.1.3. Extraction recovery

The results show a good mean extraction recovery ($n = 3$) ranging from 64 to 78% with a precision below 12% at the 4 QC concentrations (Table 3).

3.1.4. Selectivity and specificity

After injection of 6 plasma samples from patients treated for tumor diseases without vincristine, no significant interference with drugs concomitantly administered was observed.

3.1.5. Matrix effect

Matrix effect was evaluated by comparing the obtained area from a blank plasma extract spiked with vincristine at 25 ng/ml and from a direct injection of diluted stock solution at the same concentration. A ratio was calculated and typical matrix effect (mean ± standard deviation, SD) was observed at 0.81 ± 0.05 for vincristine. The moderate ion suppression observed did not have an impact on the expected sensitivity.

3.1.6. Stability

The stability of vincristine in plasma at room temperature for 15, 8 and 6 h has been previously demonstrated [6–8]. Aliquot of QC and calibrator samples were stored at −40 °C for 6 months and did not show any relevant change in signal intensities of target compounds (data not shown). Vincristine concentration in patient samples after 6 months at −80 °C or after three freeze thaw cycles did not show significant deviations (<±16%) from the nominal concentrations. Extract stability at +4 °C over 48 h, on the four QC levels, showed a maximal deviation from the nominal concentration of 7.7%.

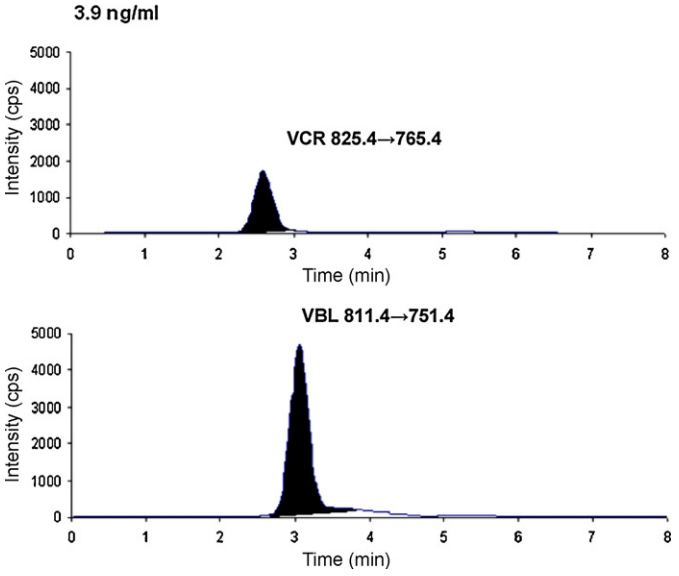

Fig. 2. Typical ion chromatogram of vincristine and internal standard (vinblastine) obtained from a patient sample with an estimated vincristine concentration at 3.9 ng/ml.

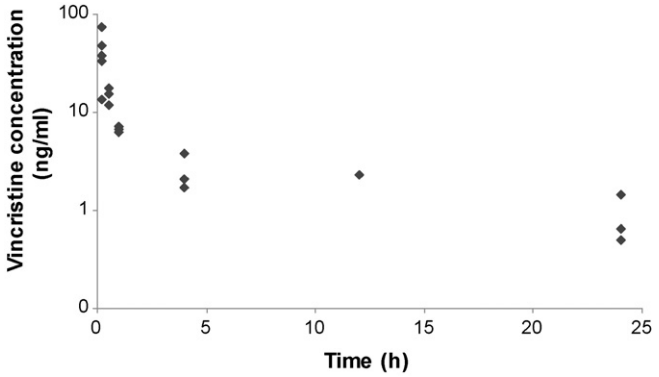

Fig. 3. Semilogarithmic vincristine concentrations versus time profile in six paediatric patients after receiving 1.2 ± 0.36 mg/m^2 of vincristine by 5 min intravenous injection.

3.2. Method application

The validated method was applied to quantify vincristine in plasma from six children ($n = 18$ samples), receiving a mean dose of 1.2 ± 0.36 mg/m^2 of vincristine by 5 min intravenous injection. A representative chromatogram of a patient plasma sample is shown in Fig. 2. The vincristine plasma concentrations versus time curve shows the characteristic biphasic elimination of this drug [11,12] with a fast initial distribution rate followed by a very slow elimination process (Fig. 3). Vincristine concentrations up to 24 h are always above the LOQ and the smaller vincristine concentration found, 24 h after the administration, was 0.50 ng/ml.

4. Conclusion

A sensitive, accurate and precise procedure based on LC/MS/MS has been developed and validated for the determination of vincristine in plasma. A good linearity was obtained over the concentration range 0.25–50 ng/ml. Moreover, this assay demonstrated a high sensitivity with a LOQ at 0.25 ng/ml using a small volume of plasma for analysis (200 μl), appropriate to quantify vincristine concentration in a paediatric population. This method was successfully applied to determine the pharmacokinetic profile of vincristine in six children. Further applications are continuing to assess vincristine pharmacokinetics in paediatric patients and to evaluate the relationship between concentration and toxicity.

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