Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 809 (2004) 273–278
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of vincristine in mouse plasma and brain tissues by liquid chromatography–electrospray mass spectrometry

Ping Guo, Xiaomin Wang, Feng Zhou, James M. Gallo*

Department of Pharmacology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111, USA

Received 16 February 2004; received in revised form 27 May 2004; accepted 17 June 2004

Available online 13 July 2004

Abstract

Vincristine is an anticancer agent that continues to be examined in preclinical models even though it is used in a variety of human neoplastic disorders. We developed a sensitive liquid chromatography–mass spectrometry (LC–MS) method for the determination of vincristine in plasma and in brain tissues that would support investigations on drug distribution into tissues in animal models. The procedure required only a small sample volume (10 μl) of plasma, which circumvented a limitation of most other assays that were developed for human samples. A solid-phase extraction procedure was employed that enabled the eluent to be directly injected onto a reversed-phase chromatographic HPLC system using positive electrospray ionization followed by mass spectrometric detection. The extraction recoveries of vincristine were 57 and 60% from plasma and brain tissues, respectively. The mobile phase consisted of methanol and 15 mM ammonium acetate in 0.02% formic acid (70:30) that was pumped at 0.2 ml/min to yield retention times of 1.6 and 1.8 min for vincristine and vinblastine, the internal standard, respectively. The method was validated at vincristine plasma concentrations from 0.01 to 2 μg/ml, and from 0.01 to 1 μg/g in brain tissue. The advantage of the method enabled the quantitation of vincristine in multiple plasma samples obtained from a single mouse, which permitted the accurate estimation of its pharmacokinetic properties.

Keywords: Vincristine

1. Introduction

Vincristine (Fig. 1) is a natural vinca alkaloid derived from the periwinkel plant *Catharantus roseus G. Don*. It has been used in variety of human neoplatic disorders for nearly 40 years. In addition to the clinical use of vincristine, it is the subject of many preclinical investigations pertaining to its interesting pharmacological properties. One area of interest is based on the fact that vincristine is a substrate for certain members of the ABC transporter family, namely, P-glycoprotein [Pgp] and multidrug resistance protein-1 [MRP1]. These membrane transporters serve as a drug efflux pumps that can alter vincristine pharmacokinetic properties and the sensitivity of tumor cells. Of particular interest to us is the brain and brain-tumor distribution of vincristine where Pgp and MRP1 function at the blood–brain barrier [Pgp], and the choroid plexus [Pgp and MRP1] to alter central nervous system penetration of drugs. Under normal physiological conditions the blood–brain barrier and the choroid plexus limit drug access to brain and cerebrospinal fluid, respectively, in part due to Pgp and MRP1. The contribution to these pumps to vincristine brain concentrations, either at the normal BBB or in the presence of a brain tumor, a condition that may compromise its function, has not been fully delineated. To facilitate the exploration of the role of these drug efflux pumps in the pharmacokinetics and brain distribution of vincristine in preclinical models, a sensitive LC–MS method was developed for small sample volumes.

Although vincristine has been used as an antineoplastic drug for nearly four decades, specific and sensitive assays suitable for its determination in plasma and the associated pharmacokinetic characteristics are limited [1–9]. There have been several HPLC methods, using either UV or electrochem-

* Corresponding author. Fax: +1 215 728 27 41.
E-mail address: jm_gallo@fccc.edu (J.M. Gallo).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.06.025

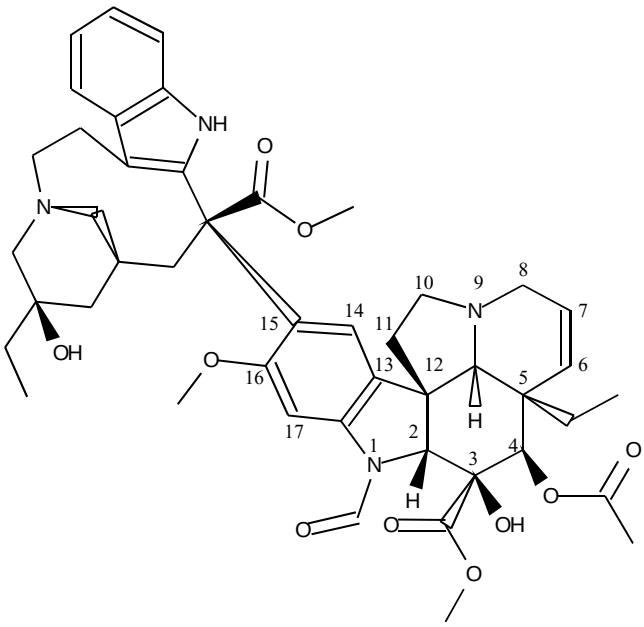

Fig. 1. Chemical structure of vincristine.

ical detection, reported for the determination of vincristine pharmacokinetic properties in biological samples [10–15]. Since vincristine lacks both strong UV absorption and electrochemical response, large volumes [0.5–4 ml] of plasma or serum are needed to achieve the desired sensitivity for pharmacokinetic studies with these methods [10,12–15]. By using UV detection at 297 nm and solid-phase extraction of 500 μl of plasma, a lower limit of quantitation of 28.6 ng/ml in plasma was achieved [10]. HPLC-electrochemical detection assays using solid-phase extraction and large sample volumes [1–1.2 ml] led to the detection of 100–300 pg of vincristine on-column at a signal-to-noise ratio of 3 [11–14]. One electrochemical based HPLC method reported a lower limit of quantitation of 0.48 ng/ml by using an online solid-phase extraction and 300 μl of plasma [11]. Although LC–MS is widely used in pharmacokinetic studies due to its high sensitivity and selectivity, there is only one report that involved liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS) to determine vincristine in human plasma [16]. This method yielded an improved sensitivity limit of 0.30 ng/ml. The method, however, required a large volume of plasma (2 ml) and involved a time consuming liquid–liquid extraction for clean up and concentration, in which 2 ml of plasma was extracted two times with 5 ml of chloroform followed by evaporation of the combined organic solvent. Drug assays that require relatively large volumes of plasma or serum may be suitable for human pharmacokinetic studies, but not for preclinical pharmacokinetic studies in rodents since the collection of large volumes of blood, due to multiple serial samples, from each animal would be prohibited. There has yet to be developed a LC–MS assay based on small sample sizes that could be applied to conducting comprehensive pharmacokinetic studies in rodents. In addition, the prevailing methods have not been applied to the determination of vincristine in brain tissue, which is a necessary extension to the methodology so that questions pertaining to vincristine disposition in brain and brain tumors can be addressed. Given the above concerns of developing a robust assay for vincristine in mouse plasma and brain tissue, our goal was to develop a sensitive LC–MS assay, which enabled the complete pharmacokinetic profile to be determined from a single mouse.

2. Experimental

2.1. Chemicals and reagents

Vincristine sulfate was purchased from Hande Tech USA Inc. (Houston, TX, USA). Vinblastine sulfate and formic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade methanol was purchased from Fisher Chemicals (Fair Lawn, New Jersey, USA). Purified water (Nanopure deionization system, Barnstead/Thermolyne, Dubuque, IA, USA) was used for all aqueous solutions. Drug-free mouse plasma was purchased from Lampire Biological laboratories (Pipersville, PA, USA). Bond Elut C2 cartridges (50 mg/1 ml) were purchased from Varian Incorporated (Harbor City, CA, USA).

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

Standard stock solutions of vincristine were prepared in methanol at a concentration of approximately 65 μg/ml and stored at −20 °C. Working solutions for brain calibration standards were prepared by diluting standard stock solutions to concentrations of vincristine ranging from 0.6 to 0.006 μg/ml with methanol. Stock solutions of the internal standard, vinblastine, were prepared at 46 μg/ml in water and stored at −80 °C. The working solutions of internal standard were prepared fresh daily by adding 5 μl stock solution of internal standard to 1500 μl of water.

Mouse plasma stock solutions of vincristine were prepared at approximately 10 μg/ml, and then used to prepare mouse plasma calibration standards at concentrations ranging from approximately 2 to 0.01 μg/ml by serial dilutions of the plasma stock solution. Calibration curves were obtained by least-squares linear regression, weighted by the reciprocal of the concentration, using the peak height ratio of vincristine to vinblastine. Quality control samples were prepared in an analogous manner at concentrations within the range of the calibration standards and were assayed in triplicate on each day that samples were analyzed.

Brain tissue homogenates were prepared by adding purified water to brain tissue at a ratio of 19:1 (ml:g) and then homogenized. Calibration standards of vincristine in brain homogenates were prepared at concentrations from approximately 0.01 to 1 μg/g by adding appropriate aliquots of the

working solutions to brain homogenate. Calibration curves were obtained by least-squares linear regression, weighted by the reciprocal of the concentration, using the peak height ratio of vincristine to vinblastine. Quality control samples in tumor homogenate were prepared in the same manner at concentrations within the range of the calibration standards and were assayed in triplicate on each day that samples were analyzed.

2.3. Solid-phase extraction

To 10 μl of plasma, 100 μl of methanol was added and vortexed for 1 min followed by centrifugation at 18,000 × *g* for 3 min. To the resultant supernatant, 20 μl of the working solution of internal standard and 900 μl of water were added, and then applied, under vacuum of less than 0.1 bar, to a C2 cartridge that was preconditioned with 1 ml methanol and 2 ml of water. The cartridge was then washed with 1 ml of water and 2 ml of 50% methanol in water followed by gradual elution of the desired components with 300 μl of a solution consisting of methanol and 15 mM ammonium acetate (70:30). Forty μl aliquots of the eluants were injected onto the LC–MS system.

For brain samples, 100 μl of homogenate was mixed with 300 μl of methanol by vortex for 1 min, and then centrifuged at 18,000 × *g* for 3 min. The resultant supernatant was mixed with 20 μl of the internal standard working solution, and 800 μl of water and then applied to a preconditioned C2 cartridge and processed as for plasma.

2.4. Method validation

The precision and accuracy of the assay was based on analyses of plasma and brain tissue samples. Plasma and brain homogenate quality control samples were included in all calibration curves and processed in triplicate. The intraday and interday means, standard deviations, % biases, and percentage coefficients of variations (%CVs) were calculated by standard methods. The limit of quantitation (LOQ) in plasma and brain tissue homogenates was defined as the lowest concentration at which the signal-to-background noise ratio was greater than 4:1, and both intraday and interday %CVs and % biases were less than 20%.

The specificity of the assay for vincristine in the presence of endogenous components of mouse was evaluated using plasma obtained from different batches of commercial mouse plasma as well as from plasma collected from the FVB mouse strain.

2.5. Liquid chromatography–mass spectrometry (LC–MS)

The HPLC system consisted of a HP model 1100 system coupled to a Finnigan Navigator Quadrupole MS. The nebulizer temperature was maintained at 150 °C and nitrogen was used as both the nebulizer gas and drying gas with flow-rate of 310 l/h. A cone voltage of 41 V and capillary voltage 4.79 V were used. The high and low mass resolutions were 1.9 and 11.5, respectively. Vincrinstine and vinblastine (internal standard) were detected at *m*/*z* values of 825 and 812, respectively with a dwell time of 0.4 s in selected-ion recording (SIR) mode with the multiplier voltage set at 600 V.

The analytical column [Phenomenex, Luna 3u C8 (2), 3 μm particle size, 50 mm × 2.0 mm], was maintained at ambient temperature. The mobile phase was prepared by mixing 350 ml of methanol and 150 ml of 15 mM ammonium acetate containing 0.02% formic acid. The flow rate was 0.2 ml/min.

2.6. Method application

The analytical method was subsequently used to analyze samples for pharmacokinetic studies in mice bearing intracerebral tumors. Briefly, FVB and mdr 1 a/b −/− mice bearing an intracerebral B-16 melanoma, an animal model we previously used for paclitaxel [17], were given vincristine at a dose of 4 mg/kg by intravenous bolus administration. Serial blood samples (20 μl) were collected from 2 to 720 min following drug administration, from which plasma (10 μl) was harvested by centrifugation and then stored at −80 °C until analysis. Brain tissues were removed at 720 min following sacrifice of the animals and stored at −80 °C until analysis. Brain tissue homogenates were prepared by adding purified water to brain tissue at a ratio of 19:1 (ml:g) and then homogenized.

3. Results and discussion

3.1. Chromatography and mass spectrometry

Vincristine and vinblastine share a very similar chemical structure, with only a minor difference at the N1 position (Fig. 1) that has either a –CHO group or $–CH_3$ group, respectively. We found that a mobile phase mixture of methanol–ammonium acetate (70:30) provided good separation of these two compounds on the Luna C8 (2) column, yet there was still a cross interference trace peak under the vincristine peak related to vinblastine. The addition of 0.02% formic acid to ammonium acetate in the mobile phase decreased the retention times of both vincristine and vinblastine, and eliminated the trace interfering peak. Under these mobile phase conditions, the resolution of vincristine and vinblastine was not complete, even though the cross interference was eliminated. However, by use of selected-ion recording (SIR) mode that allowed two channel monitoring of the mass to charge ratios at *m*/*z* 825 and at *m*/*z* 812, corresponding to vincristine and vinblastine, respectively, both compounds were appropriately quantitated. A total run time of 6 min was allowed between successive injections to avoid endogenous interfering peaks.

3.2. Extraction of vincristine from biological matrices

A variety of solid-phase materials have been used, such as BondElut CBA [10], BondElut Diol [13], BondElut CN [14], and octadecyl silane (ODS) [11,12], for the solid-phase extraction of vincristine from plasma. After trying numerous solid phases columns including many of those previously used, we found that vincristine could be strongly retained on C2 solid-phase extraction columns, which proved to be the most efficacious. Initial attempts with larger capacity [100 mg] C2 columns required large volumes of organic solvents to elute vincristine that necessitated an evaporation step to concentrate the sample. By switching to a lower capacity C2 (50 mg) column, vincristine could be eluted with a relatively small volume (300 μl) of methanol −15 mM ammonium acetate (70:30) that eliminated a solvent evaporation step. In addition, we found that treatment of the cartridges with 2 ml of 50% methanol solutions was able to elute numerous endogenous components from the biological matrices without loss of vincristine, and also reduced strong background interferences and the run time. Successful application of the C2 solid-phase extraction procedure for vincristine required pretreatment of plasma with methanol to disrupt vincristine from plasma protein binding sites. The net result of the methanol precipitation and C2 solid-phase extraction steps yielded a mean recovery from mouse plasma of 57% for vincristine over a concentration range of between 0.013 and 2 μg/ml. Analogous advantages of the extraction procedure were also realized in brain homogenate samples, in which the mean recovery was 59.9% over a concentration range of between 0.035 and 0.96 μg/g brain tissues. Higher extraction recoveries of vincristine from C2 solid phase cartridges would require the use of large elution volumes to overcome the strong retention of vincristine on these columns. Even with the use of a large elution volume, however, an evaporation step would be inevitable to concentrate the sample making the procedure less convenient. Other means to increase recovery, such as changes in the proportions of methanol to ammonium acetate did not yield improved recovery of vincristine in either plasma or brain samples. Following extraction, sample solutions of vincristine were stable for 24 h at 4 °C, while at room temperature vincristine degraded by 18–28% in 1–2 h.

3.3. Method validation

The method was validated for vincristine in mouse plasma and in brain tissue homogenates over concentration ranges of 0.01–2 μg/ml in plasma and from 0.01 to 1 μg/g in brain tissue. Calibration curves prepared over these concentration ranges were linear with average correlation coefficients greater than 0.99 in both plasma and in brain homogenates. The method yielded mean intraday and interday precision and accuracy values of less than ± 12% (Tables 1 and 2). The LOQs were 0.013 μg/ml for plasma and 0.036 μg/g for brain tissue.

3.4. Method application

Representative chromatograms of vincristine and vinblastine (internal standard) in plasma and brain tissue homogenate are shown in Figs. 2–7. The retention time of vincristine and vinblastine were 1.6 and 1.8 min, respectively with a total run time of 6 min required to avoid interfering peaks. There were no interfering peaks at the retention time of vincristine and internal standard in blank plasma and blank brain tissue samples.

Concentration–time profiles of vincristine in mouse plasma after intravenous administration of 4 mg/kg vincristine is shown in Fig. 8.

The pharmacokinetic parameters were calculated by noncompartmental analyses from the vincristine plasma concentration–time profiles. The results are summarized in Table 3. There were no significant differences ($P > 0.05$)

Table 1
Intraday precision and accuracy of vincristine in plasma and brain tissue homogenate ($n = 3$)

	Plasma (μg/ml)			Brain tissue (μg/g)			
Concentration added	0.0129	0.0738	0.421	2.01	0.0354	0.318	0.955
Concentration measured	0.0134	0.0752	0.440	2.08	0.0323	0.317	0.957
S.D.	0.0016	0.0057	0.020	0.14	0.0029	0.037	0.048
CV (%)	11.7	7.5	4.5	6.8	10.4	11.6	5.0
Bias (%)	3.9	1.9	4.4	3.5	−8.8	−0.3	0.2

Table 2
Interday precision and accuracy of vincristine in plasma and brain tissue homogenate ($n = 6$)

	Plasma (μg/ml)			Brain tissue (μg/g)			
Concentration added	0.0129	0.0738	0.421	2.013	0.0354	0.318	0.955
Concentration measured	0.0135	0.0730	0.421	2.014	0.0335	0.329	0.961
S.D.	0.0015	0.0052	0.054	0.04	0.0029	0.018	0.022
CV (%)	11.1	7.2	12.8	2.0	8.6	5.3	2.3
Bias (%)	4.7	−1.1	0.0	0.05	−5.4	3.5	0.6

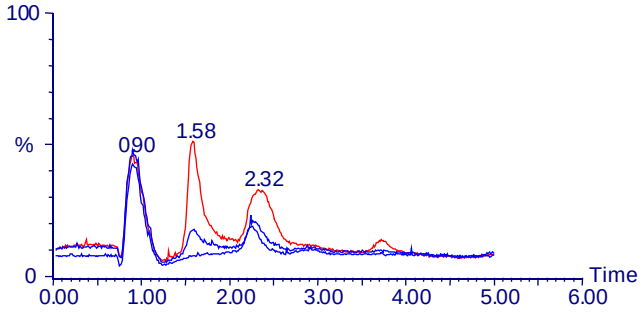

Fig. 2. Chromatograms of vincristine in mouse plasma: blank, 13 and 74 ng/ml. The retention time of vincristine is 1.6 min, monitored at *m/z* 825.

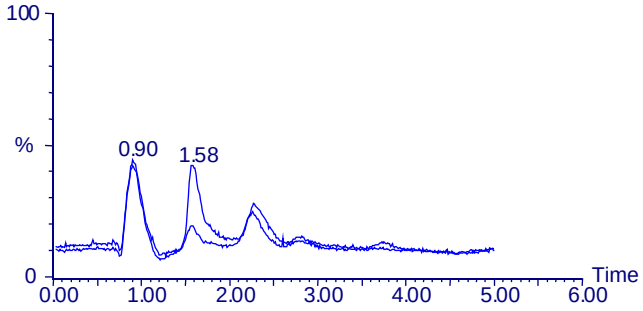

Fig. 3. Chromatograms of vincristine in mouse plasma obtained at 2 and 12 h after administration of 4 mg/kg vincristine. The retention time of vincristine is 1.6 min, monitored at *m/z* 825.

between any of the pharmacokinetic parameters (i.e. systemic clearance, half-life and volume of distribution) between the FVB wild-type and Pgp knockout [mdr 1 a/b (−/−)] mice, although the Pgp knockout mice had lower mean total clearance and longer half-life values compare to that of wild-type mice suggesting an effect on hepatobiliary clearance. Although the complete effect of Pgp on vincristine brain distribution cannot be assessed by examination of concentrations at a single time point, 720 min in this case, left brain concentrations were about two-fold higher in the Pgp knockout mice consistent with removal of its efflux function at the blood–brain barrier. Right brain and tumor vincristine concentrations at 720 min were not similarly affected. Additional studies will be needed

Table 3
Pharmacokinetic parameters and brain distribution at 720 min of vincristine after intravenous administration of 4 mg/g

Parameter[a]	4 mg/kg (FVB wild type)	4 mg/kg (mdr 1 a/b −/− mice)
CL (ml/min kg)	91.0 ± 11.6	69.2 ± 23.5
$T_{1/2\,(terminal)}$ (min)	229.9 ± 65.8	298.3 ± 84.8
Vss (ml/kg)	19192.9 ± 4025.1	19249.0 ± 6112.6
Left brain (ug/kg)	0.018 ± 0.003*	0.032 ± 0.007
Right brain (ug/kg)	0.031 ± 0.013	0.028 ± 0.011
Brain tumor (ug/kg)	0.536 ± 0.376	0.431 ± 0.165

[a] Abbreviations are: CL = total systemic clearance; Vss = volume of distribution at steady state; $T_{1/2\,(terminal)}$ = terminal elimination half-life. Values are means ± S.D.; n = 4–5.

* $P < 0.05$.

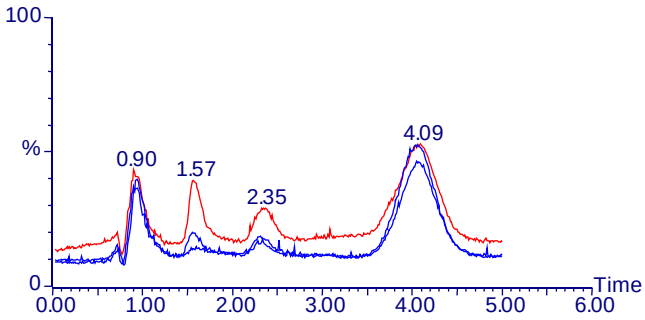

Fig. 4. Chromatograms of vincristine in brain tissue homogenate: blank, 35 and 106 ng/g. The retention time of vincristine is 1.6 min, monitored at *m/z* 825.

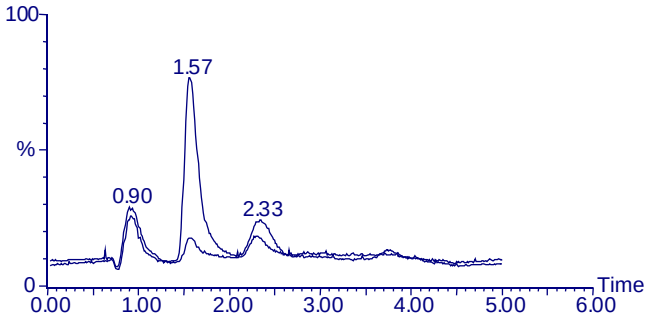

Fig. 5. Chromatograms of vincristine in mouse brain and brain tumor obtained at 12 h after administration of 4 mg/kg vincristine. The retention time of vincristine is 1.6 min, monitored at *m/z* 825.

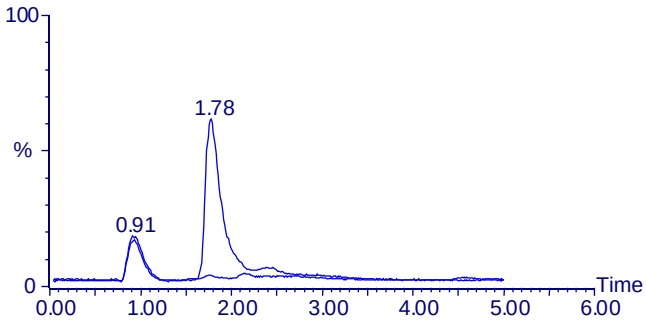

Fig. 6. Chromatograms of vinblastine (internal standard) in mouse plasma: blank, 307 ng/ml. The retention time of vinblastine is 1.8 min, monitored at *m/z* 812.

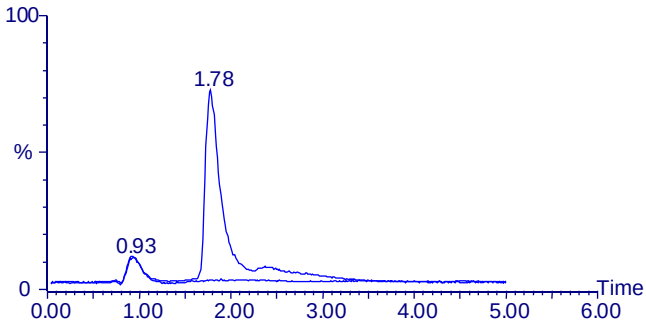

Fig. 7. Chromatograms of vinblastine (internal standard) in mouse brain: blank, 613 ng/g. The retention time of vinblastine is 1.8 min, monitored at *m/z* 812.

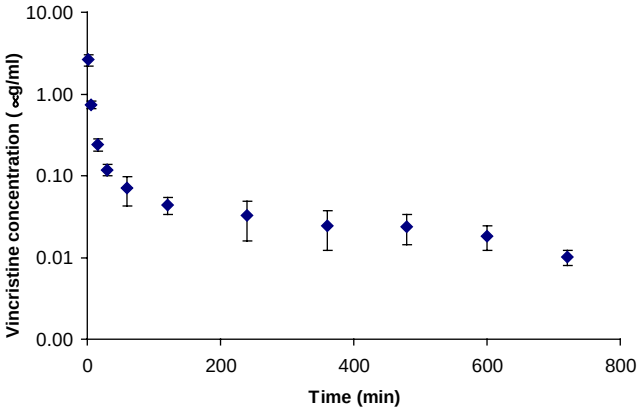

Fig. 8. Plasma concentration–time profiles of vincristine in FVB mouse plasma after administration of 4 mg/kg (n = 5) vincristine intravenously.

to fully characterize the influence of Pgp on vincristine distribution in brain tumor bearing animals.

4. Conclusion

A validated and sensitive LC–MS method for the quantitation of vincristine in mouse plasma and brain tissue is presented. The unique features of the assay were the use of very small sample volumes (10 μl plasma), and a relatively rapid extraction procedure that did not require solvent evaporation. The ability to measure vincristine in small plasma volumes facilitated the design of pharmacokinetic studies that utilized collection of serial blood samples in each mouse. The assay % biases and CVs were less than 12% and readily reached quantitation limits of 13 ng/ml of vincristine for plasma and 35 ng/g for brain tissue, respectively. The method was successfully applied in pharmacokinetic and brain tissue distribution studies in mice bearing brain tumors, a preclinical model that can be used to assess the role of P-glycoprotein on the uptake of anticancer drugs in brain and brain-tumors. The assay is convenient and should be suitable for measuring vincristine in preclinical pharmacokinetics studies.

Acknowledgements

Partial funding for this work was provided by NIH Specialized Program of Research Excellence (SPORE) in Ovarian Cancer grant, CA 83638 and NIH grant CA 72937.

References

[1] E. Groninger, T. Meeuwsen-de Boar, P. Koopmans, D. Uges, W. Sluiter, A. Veerman, W. Kamps, S. de Graaf, Pediatr. Res. 52 (2002) 113.
[2] K. Villikka, K.T. Kivisto, H. Maenpaa, H. Joensuu, P.J. Neuvonen, Clin. Pharmacol. Ther. 66 (1999) 589.
[3] S. Song, H. Suzuki, R. Kawai, Y. Sugiyama, Drug Metab. Dispos. 27 (1999) 689.
[4] C.E. Gidding, G.J. Meeuwsen-de Boer, P. Koopmans, D.R. Uges, W.A. Kamps, S.S. de Graaf, Cancer Chemother. Pharmacol. 44 (1999) 203.
[5] K.A. Gelmon, A. Tolcher, A.R. Diab, M.B. Bally, L. Embree, N. Hudon, C. Dedhar, D. Ayers, A. Eisen, B. Melosky, C. Burge, P. Logan, L.D. Mayer, J. Clin. Oncol. 17 (1999) 697.
[6] L. Embree, K. Gelmon, A. Tolcher, N. Hudon, J. Heggie, C. Dedhar, P. Logan, M.B. Bally, L.D. Mayer, Cancer Chemother. Pharmacol. 41 (1998) 347.
[7] S.S. de Graaf, H. Bloemhof, D.E. Vendrig, D.R. Uges, Med. Pediatr. Oncol. 24 (1995) 235.
[8] W.R. Crom, S.S. de Graaf, T. Synold, D.R. Uges, H. Bloemhof, G. Rivera, M.L. Christensen, H. Mahmoud, W.E. Evans, J. Pediatr. 125 (1994) 642.
[9] J.K. Horton, K.N. Thimmaiah, J.A. Houghton, M.E. Horowitz, P.J. Houghton, Biochem. Pharmacol. 38 (1989) 1727.
[10] L. Embree, K.A. Gelmon, A.W. Tolcher, N.J. Hudon, J.R. Heggie, C. Dedhar, M.S. Webb, M.B. Bally, L.D. Mayer, J. Pharm. Biomed. Anal. 16 (1997) 675.
[11] P. Koopmans, C.E. Gidding, S.S. de Graaf, D.R. Uges, Ther. Drug Monit. 23 (2001) 406.
[12] H. Bloemhof, K.N. Van Dijk, S.S. De Graaf, D.E. Vendrig, D.R. Uges, J. Chromatogr. 572 (1991) 171.
[13] D.E. Vendrig, J.J. Holthuis, V. Erdelyi-Toth, A. Hulshoff, J. Chromatogr. 414 (1987) 91.
[14] D.E. Vendrig, J. Teeuwsen, J.J. Holthuis, J. Chromatogr. 424 (1988) 83.
[15] M. De Smet, S.J. Van Belle, G.A. Storme, D.L. Massart, J. Chromatogr. 345 (1985) 309.
[16] J. Ramirez, K. Ogan, M.J. Ratain, Cancer Chemother. Pharmacol. 39 (1997) 286.
[17] J.M. Gallo, S. Li, P. Guo, K. Reed, J. Ma, Cancer Res. 63 (2003) 5114.